VOLUME XXXVI SEPTEMBER, 1957 NUMBER 9

The Journal of Clinical Investigation

EDITED FOR THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION

Contents

Depletion of Carcass Potassium in Rats Made Hypertensive With Desoxycorticosterone Acetate (DCA) and With Cortisone. ABBIE I. KNOWLTON AND EMILY N. LOEB 1295

The Physiological Disposition and Metabolic Fate of Cortisone in Man. RALPH E. PETERSON, CHARLES E. PIERCE, JAMES B. WYNGAARDEN, JOSEPH J. BUNIM, AND BERNARD B. BRODIE 1301

Thyroid-Adrenocortical Interrelations: Failure to Demonstrate Antagonism Between Triiodothyronine and Hydrocortisone in Man. KENNETH FREMONT-SMITH, FRANK L. IBER, AND IRVIN C. PLOUGH 1313

The Influence of Autonomic Vasoregulatory Reflexes on the Rate of Sodium and Water Excretion in Man. HENRY N. WAGNER, JR. 1319

Acid Mucopolysaccharides in Normal Serum. ALFRED JAY BOLLET, MARIA W. SERAYDARIAN, AND WILLIAM F. SIMPSON 1328

Thyroxine-Binding by Serum and Urine Proteins in Nephrosis. Qualitative Aspects. JACOB ROBBINS, J. E. RALL, AND MARY L. PETERMAN 1333

A Comparison of the Phenylalanine Content of the Hemoglobin of Normal and Phenylketonuric Individuals: Determination by Ion Exchange Chromatography. DAVID W. ALLEN AND W. A. SCHROEDER 1343

Physiological Disposition of 4-C^{14}-Cortisol During Late Pregnancy. CLAUDE J. MIGEON, JEAN BERTRAND, AND PATRICIA E. WALL 1350

A Simple Nephelometric Method for the Determination of Human Serum Beta-Lipoprotein. PETER BERNFELD, MORDECAI F. BERKOWITZ, AND VIRGINIA M. DONAHUE 1363

Influence of Cortisone and Prednisolone in Hyperbilirubinemia. RICARDO KATZ, HÉCTOR DUCCI, AND HERNÁN ALESSANDRI 1370

The Relationship of Total Exchangeable Potassium and Chloride to Lean Body Mass, Red Cell Mass and Creatinine Excretion in Man. F. P. MULDOWNEY, J. CROOKS, AND M. M. BLUHM 1375

The Journal of Clinical Investigation

Printed by the Lancaster Press, Inc., Prince and Lemon Sts., Lancaster, Pa. Published by the American Society for Clinical Investigation. Publication Office, 333 Cedar Street, New Haven 11, Conn.

PHILIP K. BONDY, EDITOR-IN-CHIEF

ASSOCIATE EDITORS

FRANKLIN H. EPSTEIN ALLAN V. N. GOODYER

The Editorial Committee of the American Society for Clinical Investigation

THOMAS P. ALMY, *New York, N. Y.*
ROBERT W. BERLINER, *Bethesda, Md.*
FRANCIS P. CHINARD, *Baltimore, Md.*
GEORGE L. CURRAN, *Kansas City, Kan.*
LEWIS DEXTER, *Boston, Mass.*
LEONARD P. ELIEL, *Oklahoma City, Okla.*
WARD S. FOWLER, *Rochester, Minn.*
ROBERT A. GOOD, *Minneapolis, Minn.*
JOHN B. HICKAM, *Durham, N. C.*
EDWARD C. REIFENSTEIN, JR., *Butler, N. J.*
ARNOLD S. RELMAN, *Boston, Mass.*
I. HERBERT SCHEINBERG, *New York, N. Y.*
JOHN V. TAGGART, *New York, N. Y.*
RICHARD W. VILTER, *Cincinnati, O.*
LAWRENCE E. YOUNG, *Rochester, N. Y.*

THE JOURNAL OF CLINICAL INVESTIGATION is designed for the publication of original investigations dealing with or bearing on the problems of disease in man, and it is the policy of the editors that the JOURNAL should cover the field of clinical investigations in its broadest sense.

Contributions should be sent to the **Editor-in-Chief, Dr. Philip K. Bondy, 333 Cedar St., New Haven 11, Conn.** In order to hasten the process of reviewing and editing it is requested that each original manuscript be accompanied by a duplicate copy and duplicate illustrations.

In order further to expedite the earliest possible publication of articles submitted to the JOURNAL OF CLINICAL INVESTIGATION they will be published in the order of final processing after review, correcting and editing rather than in the strict sequence of their receipt in this office. Papers which require much editorial change, revision of charts and tables, condensation and rewriting will be delayed according to the time required for their correction. This policy will put a premium on excellence of manuscript as well as the major consideration of content. Because of the increasing costs of printing, especially in the hand work of making tables, the editors suggest that when feasible photographs of tables be prepared. It is also requested that charts be photographed, the photographs accompanying the manuscript. The originals of the charts need not be forwarded except by special request of the editors. Further information regarding the preparation of manuscripts may be obtained from the editors.

Each volume will consist of 12 monthly issues appearing throughout the calendar year. The subscription price is $15 per volume, **payable in advance;** foreign countries also $15, payable in U. S. funds. Single copies will be sold when available at Vols. 1–28, $2; Vols. 29–36, $1.50 a copy. Authors may purchase reprints at scheduled prices. **A special subscription rate of $6 per volume of 12 monthly issues is offered to medical students, interns, fellows, and residents of approved hospitals.** Subscriptions are **net postpaid.** Remittance should be made by draft, check or postal money order, payable to **The Journal of Clinical Investigation, 333 Cedar Street, New Haven 11, Conn.** Claims for copies lost in the mails must be received within **thirty** days (domestic), **ninety** days (foreign), of the date of issue. Changes of address must be received at least two weeks in advance of issue.

Acceptance for mailing at special rate of postage provided for in paragraph (d-2), section 34.40, P.L. & R. of 1948, authorized February 13, 1950

Entered as second-class matter September 16, 1931, at the Post Office at Lancaster, Pa., under the Act of March 3, 1879

Copyright © 1957, American Society for Clinical Investigation

Nuclear-Chicago instruments being used are new 132 Analyzer Computer and new DS5-5 Well Counter.

diagnosis of pernicious anemia with radiocobalt vitamin b_{12}

This newly-designed equipment provides an ideal combination for the widely used Schilling test. Low background and high efficiency for Co^{60} gamma rays permit direct measurement of urinary excretion of radiocobalt labelled vitamin B_{12}. Concentration of urine is no longer necessary with acceptable doses of radioactivity. A unique timing device gives results directly in percent of administered dose present in urine without calculation.

This combination is also ideally effective for estimation of plasma volume with iodinated human serum albumin, red cell mass and red cell survival with radiochromate labelled erythrocytes, and other procedures requiring liquid sample counting.

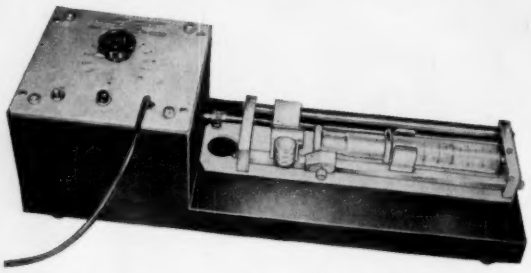
For years Nuclear-Chicago has been a leader in the development of quality radioactivity instrumentation for the medical profession, providing in the highest degree the efficiency, accuracy and reliability so essential in this exacting field. Competent and prompt service is always available. Write for full details or ask to have our representative call.

References: 1. R. F. Schilling, D. V. Clatanoff and D. R. Korst: J. Lab. Clin. Med. *45:* 926-934 (1955). 2. J. R. Krevans, C. L. Conley and M. V. Sachs: J. Chronic Dis. *3:* 234-252 (1956).

Radioisotopes are creating new frontiers in medicine

nuclear-chicago
CORPORATION
269 WEST ERIE STREET • CHICAGO 10, ILLINOIS

INFUSION/WITHDRAWAL PUMP



Catalog No. 600-900

SPECIFICATIONS

- 12-speed gear box with synchronous motor
- Accepts 50, 30, 20, 10, 5 and 2 cc standard syringes
- 72 Rates from 38.2 cc/minute to .0002 cc/minute using various size syringes
- Capable of reproducing a given rate to ± .01%
- Speeds can be varied while in operation
- Simple automatic limit disconnect
- Reversing switch converts to withdrawal pump intended for use with dye dilution apparatus
- Fixed speed models available
- Small compact size: 22″ × 7″ × 7″
- Stainless steel lead screw

$270.00—f.o.b. Dover, Mass.

THE HARVARD APPARATUS CO., INC., Dover, Massachusetts, U.S.A.

(A nonprofit institution)

6,000 CLINICAL STUDIES

Automatic data processing machines helped to speed the evaluation of clinical results from 6,000 diabetics on Orinase.* Cases were evaluated on the basis of 29 criteria.

*Trademark, Reg. U.S. Pat. Off.—tolbutamide, Upjohn

THE UPJOHN COMPANY
Kalamazoo, Michigan

Upjohn

WANTED

Back Issues of

THE JOURNAL OF CLINICAL INVESTIGATION

to fill out incomplete volumes:

January, July, (Pt. I), September, and December, 1955

January through May, 1956

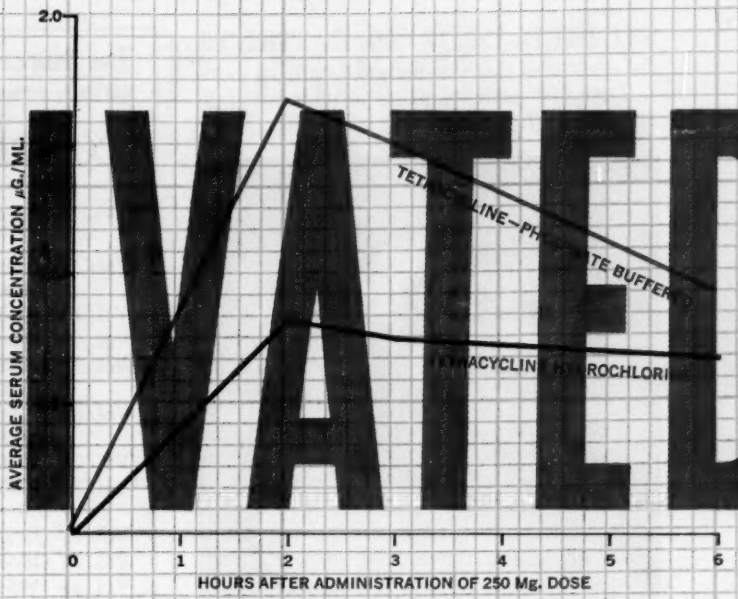
75¢ per copy and postage

For particulars write to

The Journal of Clinical Investigation
Business Office
333 Cedar Street
New Haven 11, Conn.

PFIZER LABORATORIES, *Division, Chas. Pfizer & Co., Inc.*, Brooklyn 6, N. Y. Pfizer

ACTIVATED



for higher, faster blood levels of tetracycline

Tetracyn V, tetracycline-phosphate buffered, given orally, produces "markedly higher serum concentrations than those obtained with tetracycline hydrochloride."[1]

In a crossover study, Tetracyn V afforded serum levels higher than tetracycline hydrochloride at two, three and six hours, following oral administration.[1]

Therapy with activated Tetracyn V thus provides a higher, faster activity level of tetracycline, established as outstanding in effectiveness and safety among broad-spectrum antibiotics.

SUPPLIED: Capsules, each containing tetracycline equivalent to 250 mg. tetracycline HCl, with added sodium metaphosphate.

1. Welch, H.; Lewis, C. N.; Staffa, A. W., and Wright, W. W.: Antibiotic Med. & Clin. Therapy 4:215 (April) 1957.

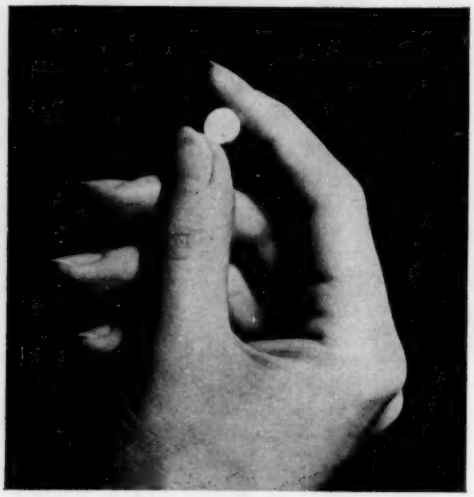
new

TETRACYN® V

tetracycline--phosphate buffered

STUDIED IN 18,000 PATIENTS

Orinase* was used investigationally in more than 18,000 patients prior to its release on June 3, 1957.



*Trademark, Reg. U.S. Pat. Off.—tolbutamide, Upjohn

THE UPJOHN COMPANY
Kalamazoo, Michigan

Upjohn

The Journal of Clinical Investigation
333 Cedar Street — New Haven 11, Conn.

COMPLETE VOLUMES OF BACK ISSUES

Vols. 1 through 11, inc.—special price	$25.00
Vol. 12 (1933) six issues	10.00
Vol. 13 (1934) six issues	10.00
Vol. 27 (1948) six issues and one supplement	10.00
Vol. 28 (1949) six issues and two supplements	10.00
Vol. 29 (1950) twelve issues	10.00
Vol. 30 (1951) twelve issues and one supplement	12.00
Vol. 31 (1952) twelve issues	12.00
Vol. 32 (1953) twelve issues	12.00
Vol. 33 (1954) twelve issues	12.00
Vol. 34 (1955) twelve issues and one supplement	12.00
Index, Vols. 1–17, inc.	2.00

Order While Available

REVUE FRANÇAISE D'ÉTUDES CLINIQUES ET BIOLOGIQUES

SEPTEMBER, 1957
Vol. 2, No. 7

Editorial

Etiologie, pathologie et pathogénie des épilepsies du lobe temporal. H. GASTAUT.

Original Articles

Introduction à la théorie mathématique de la physiologie hépatique. P. VENDRYES.

Phénomènes électrocorticaux provoqués chez l'animal par la stimulation d'afférences somesthésiques. R. MARTY, J. SCHERRER et F. CONTAMIN.

Les échanges de sodium durant l'hypothermie. Etude expérimentale à l'aide du radio-sodium chez le lapin. F. MOREL et A. COMBRISSON.

Activité de certaines protéines de la coagulation dans le plasma et les urines au cours du syndrôme néphrotique. H. YATZIDIS et G. RICHET.

Facteurs de l'hyperaldolasémie au cours des myopathies. F. SCHAPIRA, J. DEMOS, G. SCHAPIRA et J. C. DREYFUS.

Technical Note

Technique de recherche des auto-hémolysines immunologiques. J. DAUSSET, J. COLOMBANI et J. EVELIN.

Subscriptions, U.S.A. and Canada, $19.00
Special rate for interns and residents, $13.00

Flammarion, 1243 University Street, Montreal

a new concept in diagnosis

now! *more rapid differential diagnosis of:*

HEPATOBILIARY DYSFUNCTION:
- common duct stones
- stricture of the common bile duct
- biliary stasis
- fibrosis of the sphincter of Oddi

OBSTRUCTIVE JAUNDICE

OSTEITIS DEFORMANS (Paget's disease)

CANCER OF BONE

CANCER METASTASIS to the liver

IMPENDING LIVER DAMAGE induced by drugs such as:
- tranquilizers
- methyltestosterone
- arsenic

---

no instruments needed

nontechnical help can be taught in 10 minutes

results in 30 minutes or less

PHOSPHATABS* (alkaline) *with* TESWELLS*

AN ORIGINAL DEVELOPMENT OF WARNER-CHILCOTT RESEARCH

for office and laboratory screening of alkaline phosphatase levels in nonicteric and icteric patients

---

TO USE:

1. Place one tablet (PHOSPHATABS) in the special test tube.
2. Add 4 drops of serum or plasma.
3. Crush tablet with wooden applicator stick.
4. Let stand for 12-30 minutes depending on room temperature (determine time from curve in PHOSPHATABS package insert).
5. Add one drop of PHOSPHATABS COLOR DEVELOPER.
6. Compare color with color chart.

PHOSPHATABS* are available in a kit containing enough reagent tablets, color developer and TESWELLS* (controlled diameter tubes) for 48 determinations . . . $15. Phosphatabs and Teswells are available from your nearest laboratory supply distributor.

*TRADEMARK

For more information write to

Laboratory Supply Division

WARNER-CHILCOTT

MORRIS PLAINS, N. J.

12,000,000 TEST TABLETS

During 16 months of clinical testing, more than 12,000,000 Orinase* tablets were made available to physicians for trial in selected diabetic patients.

*Trademark, Reg. U.S. Pat. Off.—tolbutamide, Upjohn

THE UPJOHN COMPANY
Kalamazoo, Michigan

Upjohn

Reprints of the eight papers on the

NEPHROTIC SYNDROME

published in the April, 1954 issue of

THE JOURNAL OF CLINICAL INVESTIGATION

are available for $1.00

Single copies of

THE TREATMENT OF ACUTE INFECTIOUS HEPATITIS

July, 1955, Part Two issue of

THE JOURNAL OF CLINICAL INVESTIGATION

$1.50

(No Reprints available)

Order both through the Business Office of

The Journal of Clinical Investigation

333 Cedar Street New Haven 11, Conn.

Thirst, too, seeks quality

...for sharp, clear waveforms

in physiological event monitoring

HERE is a high quality, easy to operate oscilloscope designed *especially* for visually monitoring physiologic waveforms in conjunction with Sanborn direct writing recording instruments. Waveform is presented as a sharp, distinct yellow trace on the Viso-Scope's double-coated 5″ diameter screen. Long image retention allows complete visual presentation of an event that lasts four seconds. Electrically analogous (in part) to a television receiver, the Viso-Scope is just as easy to operate: sweep speed (25, 50 and 100 mm/sec.) is selected by turning a knob . . . three other knobs control on-off and Scale Brightness, Focus, and Trace Brightness. These are the only controls needed for routine operation.

Usefulness of the Viso-Scope is further enhanced by: provision for enlarging the tracing at any time to a scale of 1″ deflection on scope for 1 cm. stylus deflection on chart; an illuminated reference scale on the screen, for estimating the size and duration of the trace; suitability to photography, with Dumont, Fairchild or other cameras; and use with other Sanborn instruments, such as the Model 179 Electronic Switch (shown below) for simultaneous presentation of as many as *four* waveforms, or with the Model 185 Vector Amplifier for vectorcardiography.

SANBORN

MODEL 169A

Viso-Scope

Detailed descriptive four-page folder, showing Viso-Scope uses with other Sanborn instruments, is available on request. Write to

SANBORN COMPANY

MEDICAL DIVISION

175 WYMAN ST., WALTHAM 54, MASS.

when infection
strikes the respiratory tract . . .

ILOTYCIN

(Erythromycin, Lilly)

provides singularly effective antibiotic therapy because

- Virtually all gram-positive organisms are sensitive
- Allergic reactions following systemic therapy are rare
- Bactericidal action kills susceptible organisms
- Normal intestinal flora is not appreciably disturbed

Dosage: The usual adult dose is 250 mg. every six hours.

Available in specially coated tablets, pediatric suspensions, drops, otic solution, ointments, and I.V. ampoules.

ELI LILLY AND COMPANY • INDIANAPOLIS 6, INDIANA, U. S. A.

732150

DEPLETION OF CARCASS POTASSIUM IN RATS MADE HYPERTENSIVE WITH DESOXYCORTICOSTERONE ACETATE (DCA) AND WITH CORTISONE

By ABBIE I. KNOWLTON and EMILY N. LOEB

(From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Presbyterian Hospital, New York, N. Y.)

(Submitted for publication March 29, 1957; accepted May 13, 1957)

The hypertension which can be induced experimentally in rats by the administration of desoxycorticosterone acetate (DCA) differs from that which develops in rats given cortisone acetate in its dependence upon a liberal sodium intake (1). Not only is DCA-hypertension made more severe by providing the animals with excess sodium (2), but it fails to develop if the available supply of this cation be sufficiently restricted (3). In contradistinction, the capacity of cortisone (1) or of hydrocortisone (4) to elevate the blood pressure is independent of sodium intake.

It seemed of interest, therefore, to determine whether the mechanisms underlying the dissimilar actions of these two groups of steroids would be reflected in the electrolyte composition of the tissues of animals given one or the other compound under conditions of sodium loading as well as of sodium restriction. A preliminary study analyzing pooled samples of tissue indicated that the hypertension induced by DCA was accompanied by an increase in carcass sodium, whereas this did not obtain in the carcasses of rats made hypertensive with cortisone (5).

METHODS

Sixty young female Sprague-Dawley rats [1] were divided at random into eight groups, averaging 118 to 135 Gm. in body weight. The allocation of the animals and the experimental regimens employed are shown in Table I. The sodium content of the "low Na" diet on analysis was somewhat higher, and that of the "high Na" diet somewhat lower, than in the preliminary study. The latter was an intentional modification introduced in the hope of avoiding the high mortality in the DCA-injected group encountered in the preliminary study. DCA [2] was administered in sesame oil, subcutaneously, six days weekly, and cortisone acetate in aqueous suspension [3] was similarly given. It was necessary to sacrifice the rats of Group VII from four to seven days after the start of the experiment, as they became moribund from adrenal insufficiency. In this brief period all had lost significant amounts of weight, and their blood pressure readings had decreased markedly or were unobtainable.

Blood pressures were measured according to the method of Friedman and Freed (6) but with the modification that the animals were neither immobilized with curare nor anesthetized. Normal values obtained by this modified technique range 10 to 20 mm. Hg higher than those obtained on anesthetized animals. Readings in Group VII were obtained daily; in other groups readings were made weekly. At sacrifice all animals were anesthetized with Nembutal®, then exsanguinated from the incised heart. Blood obtained in this manner from individual rats was separated, and the sera combined with that of two or three other animals to form two pools per group. On these pooled sera, sodium and potassium were determined using a flame photometer with an internal lithium standard; and chloride by the Van Slyke method as modified by Wilson and Ball (7).

After removal of the gastrointestinal canal, heart, kidneys, right gastrocnemius, right femur and a 1-cm. square sample of skin, the remaining carcass was frozen.

The individual frozen carcasses were coarsely ground by hand in a small meat grinder, and the water content determined on a 20-Gm. aliquot after desiccation for two weeks in an electric oven at 90° to 105° F. Fat content was determined on this dried tissue following a 24-hour extraction with ethyl ether in a Soxhlet apparatus. The dry defatted tissue was then ground in a mortar and aliquots were taken for sodium, potassium, chloride and nitrogen determinations. For sodium and potassium, 0.4 to 0.5 Gm. of tissue were placed in a chloride tube and digested 20 to 30 minutes in a water bath at 80° to 90° C. with 5 ml. nitric acid, then diluted to approximately 30 ml., filtered twice through washed glass wool and made up to 100 ml. volume with 1:200 parts of lithium by weight. Determinations were made in a Baird flame photometer. No corrections for calcium were made, as the amount present in samples of tissue was found insufficient to interfere with the sodium determinations. Similarly, no correction was made for nitric acid, since the small amount of lowering which this substance

[1] Obtained from the Holtzman Co., Madison, Wisconsin.

[2] We wish to thank Dr. Kenneth Thompson of Organon, Inc., Nutley, N. J., for generously supplying the desoxycorticosterone acetate.

[3] Kindly made available by Merck & Co., Rahway, N. J.

TABLE I

Allocation of animals

Group	Diet	Drinking water	Adrenals	Steroid	Experimental period
				mg./day	*days*
I	High Na*	0.85% NaCl	Excised	DCA 2.5	21
II	High Na	0.85% NaCl	Excised	Cortisone Ac. 2.5	21
III	High Na	0.85% NaCl	Excised	nil	21
IV	High Na	0.85% NaCl	Intact	nil	21
V	Low Na†	Tap water	Excised	DCA 2.5	14
VI	Low Na	Tap water	Excised	Cortisone Ac. 2.5	14
VII	Low Na	Tap water	Excised	nil	4–7
VIII	Low Na	Tap water	Intact	nil	14

* On analysis Na content = 0.6 per cent.
† On analysis Na content = 0.1 per cent.

produces in both the sodium and potassium readings was found to be within the limits of error of the method. For chloride, 0.5-Gm. samples were placed in chloride tubes and the same method employed as for sera, with the exception that samples were allowed to stand 24 hours after addition of the silver nitrate and nitric acid before proceeding with heating and titration with ammonium thiocyanate. For nitrogen, a macro-Kjeldahl digestion was carried out on 1-Gm. samples. Following digestion and dilution with 130 ml. water, the digest was

TABLE II

Comparison of effects of DCA vs. cortisone acetate (E) on body weight, blood pressure and serum electrolytes of adrenalectomized rats

Regimen	No. rats	Change in weight *Gm.*		Final blood pressure *mm. Hg*		Serum*		
						Na^+	K^+	Cl^-
						mEq./L.		
		Aver.	S.D.	Aver.	S.D.			
High Na diet, 21 days								
I DCA + Adrx.	8	+46	3.6	*189*†	10	142.6 143.8	*3.8* *3.5*	*85.3* *87.1*
II E + Adrx.	7	−14	5.9	*195*	2	143.2 142.8	4.8 5.0	96.5 97.6
III Adrx.	6	+31	10.2	*132*	12	134.8 127.6	5.9 6.7	96.1 89.5
IV Intact controls	6	+36	8.4	149	10	136.2 138.2	5.5 5.9	97.3 100.0
Low Na diet, 14 days								
V DCA + Adrx.	8	+28	10.5	137	6	136.0 139.0	6.9 6.6	95.4 95.0
VI E + Adrx.	7	−26	8.5	*195*	17	140.0 140.0	5.2 5.0	*93.8* *94.5*
VII Adrx.	7	−21	6.4	*87*‡		*125.2* *120.6*	*9.8* *9.0*	
VIII Intact controls	6	+32	8.1	150	9.2	139.0 140.8	5.7 5.4	95.8 96.7

* Each serum value = determination made on pooled sera from 3 to 4 individual rats.
† Treatment differences in blood pressures were jointly estimated by a 95 per cent simultaneous confidence interval following Tukey (8). (For the individual treatment comparisons this amounts to significance tests more strict than at the 5 per cent level.) Blood pressure values which differed significantly from intact controls on the same regimen have been italicized. For each of the serum electrolytes, treatment differences were based on weighted averages of the pool values and compared again by Tukey's method. Whenever the weighted averages differed significantly from intact controls, the separate determinations were italicized.
‡ Rats sacrificed after 4 to 7 days, at which time blood pressure obtainable in only one animal in the group.

filtered and made to a 250-ml. volume. The nitrogen content of a 2-ml. aliquot was then determined after alkalinization by steam distillation of the resulting ammonia, trapped in boric acid and titrated against N/50 HCl.

RESULTS

The results are set forth in Tables II and III.

Weight

In the groups provided with liberal quantities of sodium, essentially normal growth was evidenced by all but the cortisone-injected rats. The impressive weight loss here was consistent with our previous observations (1) and again emphasizes the marked growth-suppressing activity of this steroid in the rat.

Even more striking weight losses were observed in the cortisone-treated group on sodium restriction, whereas the steroid DCA, even under these conditions of limited intake of sodium, permitted normal growth. With no steroid replacement (Group VII) a very severe weight loss was seen even in the brief period of four to seven days during which these animals survived.

Blood pressure

As anticipated, the hypertensive action of DCA was evident only among those rats receiving liberal amounts of sodium, whereas equally severe degrees of hypertension were seen on the low as well as the high sodium intake among the animals given cortisone.

Electrolytes in sera

DCA given with liberal quantities of sodium resulted in a significant lowering of the serum potassium and chloride. The serum sodium, when compared to the control group, appeared elevated. When sodium intake was restricted the steroid was a sufficiently potent sodium retainer to maintain a normal serum sodium concentration, but under these conditions no reduction in potassium or chloride occurred. Indeed, the potassium determinations on this regimen were higher than those of control animals.

Unlike DCA, cortisone did not lead to hypochloremia or a reduced serum potassium when given to animals on a high sodium regimen. However, like DCA, cortisone did permit the maintenance of a normal serum sodium concentra-

TABLE III

Comparison of effects of DCA vs. cortisone acetate (E) on carcass composition of adrenalectomized rats

Regimen	No. rats	Terminal body wt. Gm.		Carcass content											
				Water %		Fat % dry wt.		Na^+		K^+ FFDT* mEq./100 Gm.		Cl^-		Nitrogen FFDT* Gm. %		Nitrogen FFWT† Gm. %	
		Aver.	S.D.	Aver.	S.D.	Aver.	S.D.	Aver.	S.D.	Aver.	S.D.	Aver.	S.D.	Aver.	S.D.	Aver.	S.D.
High Na—21 days																	
I DCA + Adrx.	8	181	14	66.4	1.3	23.8	3.4	*20.4*‡	1.3	*21.1*	1.2	10.9	1.0	11.3	0.5	2.79	0.04
II E + Adrx.	7	121	12	*63.0*	1.3	23.1	3.1	17.9	1.0	*23.0*	1.5	11.6	0.8	10.9	0.6	*3.11*	0.03
III Adrx.	6	166	15	*69.4*	1.1	*13.3*	1.9	16.8	1.4	25.9	1.9	12.3	1.1	11.2	0.3	*2.73*	0.14
IV Intact controls	6	159	9	65.8	1.2	20.8	4.7	17.3	1.0	25.6	1.1	11.5	0.8	11.2	0.5	2.91	0.04
Low Na—14 days																	
V DCA + Adrx.	8	150	11	*69.3*	1.2	*13.5*	2.7	*15.6*	1.0	26.5	2.3	11.2	0.5	10.9	0.4	2.78	0.09
VI E + Adrx.	7	105	5	*64.2*	1.0	*12.8*	5.5	14.4	0.8	*21.5*	2.4	8.1	0.4	9.7	0.5	*3.26*	0.01
VII Adrx.§	7	104	9	*70.1*	1.5	*11.4*	6.0	13.9	0.7	27.0	1.6	9.9	0.2	11.7	0.5	2.72	0.05
VIII Intact controls	6	150	16	66.9	0.8	22.8	3.4	13.8	0.5	25.1	1.3	10.1	0.2	11.0	0.5	2.76	0.07

* Fat-free dry tissue. † Fat-free wet tissue.

‡ For each category of measurements, the treatment differences were jointly estimated by a 95 per cent simultaneous confidence interval following Tukey (8). (This amounts to testing the individual treatment differences more strictly than at the 5 per cent level.) Values for water, fat, Na^+, K^+, Cl^- and nitrogen which differed significantly from intact controls on the same regimen have been italicized.

§ Rats sacrificed after 4 to 7 days.

tion even in the face of a restricted intake. On this regimen the serum chloride was lower than normal and the serum potassium appeared lower than in the DCA groups. This latter difference may be a reflection of the different effect of these two steroids in sodium restricted rats upon carcass potassium (see below).

In contrast to both steroid injected groups, the untreated adrenalectomized rats developed striking abnormalities within four to seven days of sodium depletion. In this study, these abnormalities were partially but not completely corrected by the provision of a moderately high sodium intake (see Group III).

Carcass analyses

DCA groups. The hypertensive DCA rats given liberal quantities of sodium showed an impressive increase in their carcass sodium along with a decrease in potassium. The water content was not significantly greater than that of normal rats and, indeed, was definitely less than that of adrenalectomized rats given no steroid replacement but maintained on a similar high sodium regimen. Since the increase in carcass sodium was not accompanied by an increase in water content or chloride content nor by a decrease in nitrogen, calculated as per cent of fat-free fresh tissue, the increase in sodium in this DCA-injected group is not the result of an extracellular edema; rather, it reflects an intracellular exchange of sodium for potassium. In the DCA-injected animals which remained normotensive on the low sodium regimen, the only electrolyte change as compared to the normal group was a slight increase in carcass sodium, which was accompanied by an increase in water content but not by a reduction in nitrogen as related to wet weight.

Cortisone acetate (E) groups. The striking changes in all hypertensive cortisone injected rats were dehydration and depletion of body potassium. These changes obtained equally in both high and low sodium intake groups. Unlike the hypertensive DCA animals, there was no reciprocal increase in carcass sodium, *i.e.*, the potassium effects here were not secondary to the changes in sodium. In fact, the carcass sodium values in both groups of cortisone-treated rats were not significantly different from those encountered in the intact controls on the respective regimens.

Control adrenalectomized groups. The impressive change observed in these animals on the low as well as those on the high sodium regimen was the increased total body water. It appears that the electrolyte content of the carcasses of the adrenalectomized rats on a high sodium intake was not significantly different from that of normal animals on a similar regimen. On the low sodium regimen a comparison of the adrenalectomized with the intact control group is misleading and probably not justified since the carcasses of the former reflected only four to seven days of sodium restriction, whereas those of the normals were analyzed after a full fourteen days on this regimen.

DISCUSSION

While the foregoing data point up certain differences in carcass composition between the rats injected with DCA and those which received cortisone acetate, it is clear that all these hypertensive rats exhibited one abnormality in common, *i.e.*, a striking lowering in the total body potassium. This obtained regardless of whether the rise in blood pressure followed DCA or cortisone. Whether this change in potassium is an integral feature in the capacity of adrenal cortical steroids to produce hypertension remains to be determined. Certainly other situations which give rise to potassium deficiency may occur without hypertension.

In the DCA-treated rats a liberal quantity of dietary sodium was necessary for both the rise in blood pressure and the potassium change to become evident. Indeed, the latter change was roughly reciprocal to the increase in carcass sodium. This observed increase in sodium and decrease in potassium is quite in keeping with previously reported changes in skeletal muscle (9–12) of DCA-treated animals, and also compatible with reports of an increase in arterial wall sodium (13) and of a decrease in brain potassium (14). The increase in sodium in the DCA rats of the current experiment is also similar to findings encountered in hypertension due to other causes: 1) an increase in the sodium content of aortae (13), of brain, heart, liver, gut, muscle, skin and spleen (15) and in total carcass (16) in experimental renal hypertension, and 2) an increase in exchangeable sodium (17) and in the sodium content of aortae (18) of humans with severe hypertensive disease.

However, the increased carcass sodium in the present experiment cannot be considered a reflection of extracellular edema as has been the case in certain instances of renal hypertension (19, 20), since these DCA rats did not have an accompanying increase in total body water or chloride, nor did they exhibit a significant decrease in carcass nitrogen expressed as per cent of fat-free wet weight. The increased sodium in this instance is better explained by assuming that an exchange of sodium for potassium has occurred intracellularly.

Although the hypertensive DCA rats all exhibited an increase in carcass sodium, this change cannot be considered essential for the development of a rise in blood pressure *per se*, since it did not occur in either group of hypertensive cortisone-injected rats. The non-essential character of the increase in sodium in hypertension has been demonstrated previously by Grollman (21) in rats made hypertensive by ten days of a choline-deficient diet during the third and fourth week of life. In these animals he was unable to find any variation from normal in sodium, potassium, magnesium, chloride or water content of brain, gut, heart, liver, skeletal muscle or skin.

The depletion of carcass potassium observed in the cortisone-injected rats in the current experiment has not, to our knowledge, been reported previously, although it is well recognized that this steroid leads to potassium diuresis in balance studies. Davis, Bass and Overman (12), who observed diuresis of potassium in cortisone- treated dogs, found that tissues from such animals, with intact adrenals, showed potassium depletion in muscle and brain but not in liver, heart, ileum, spleen, lungs or skin. An increase in sodium accompanied the potassium changes in muscle.

The dehydration and the electrolyte changes observed in our studies in the rat cannot be attributed to the marked growth-inhibiting action of the steroid, since no such changes were found in normal rats starved so as to achieve a comparable weight loss (5). This dehydration of the cortisone-treated animals is impressive and may possibly reflect over-activity of the mechanism which regulates water excretion. In contrast there was overhydration in the adrenalectomized rats given no steroid supplement. In the salt depleted group (Group VII) this overhydration was clearly intracellular and is compatible with similar changes reported previously in muscle (22, 23). Over-hydration was also evident in the companion group (Group III) afforded a liberal sodium intake, though in this instance, the decrease in the nitrogen values expressed as per cent of fat-free wet weight makes it likely that some portion of the excess fluid was stored extracellularly.

SUMMARY

The data demonstrate that the carcasses of rats made hypertensive by injection of DCA or of cortisone acetate contain significantly less potassium than normal.

The potassium decrease in the carcasses of rats made hypertensive with DCA is accompanied by a reciprocal increase in carcass sodium. Sodium restriction prevented both the hypertension and, to a large extent, the electrolyte abnormalities.

The potassium decrease in the carcasses of rats made hypertensive with cortisone is not accompanied by a reciprocal change in sodium and develops under conditions of sodium restriction as readily as on a high sodium intake. Whether this correlation between potassium depletion and hypertension has causal significance cannot be stated.

The marked dehydration present in adrenalectomized rats given cortisone and the striking overhydration present in adrenalectomized control rats were equally apparent on high and low sodium intakes.

ACKNOWLEDGMENT

The authors are indebted to Dr. Agnes Berger and Miss Sheila Smythe, of the School of Public Health and Administrative Medicine of the Faculty of Medicine, Columbia University, for statistical advice in the analysis of the data.

The authors also wish to express their gratitude to Mrs. Josephine Hoban and Mrs. Tania Budzilovich for their expert technical assistance.

REFERENCES

1. Knowlton, A. I., Loeb, E. N., Stoerk, H. C., White, J. P., and Heffernan, J. F., Induction of arterial hypertension in normal and adrenalectomized rats given cortisone acetate. J. Exper. Med., 1952, **96**, 187.
2. Selye, H., Hall, C. E., and Rowley, E. M., Malignant hypertension produced by treatment with desoxycorticosterone acetate and sodium chloride. Canad. M. A. J., 1943, **49**, 88.

3. Knowlton, A. I., Loeb, E. N., Stoerk, H. C., and Seegal, B. C., Desoxycorticosterone acetate, the potentiation of its activity by sodium chloride. J. Exper. Med., 1947, **85**, 187.
4. Friedman, S. M., Friedman, C. L., and Nakashima, M., The hypertensive effect of compound F-acetate (17-OH-corticosterone-21-acetate) in the rat. Endocrinology, 1952, **51**, 401.
5. Knowlton, A. I., Studies on the hypertensive action of adrenal steroids, In press.
6. Friedman, M., and Freed, S. C., Microphonic manometer for indirect determination of systolic blood pressure in the rat. Proc. Soc. Exper. Biol. & Med., 1949, **70**, 670.
7. Wilson, D. W., and Ball, E. G., A study of the estimation of chloride in blood and serum. J. Biol. Chem., 1928, **79**, 221.
8. Tukey, J. W., Comparing individual means in the analysis of variance. Biometrics, 1949, **5**, 99.
9. Miller, H. C., and Darrow, D. C., Relation of serum and muscle electrolyte, particularly potassium, to voluntary exercise. Am. J. Physiol., 1941, **132**, 801.
10. Ferrebee, J. W., Parker, D., Carnes, W. H., Gerity, M. K., Atchley, D. W., and Loeb, R. F., Certain effects of desoxycorticosterone. The development of "diabetes insipidus" and the replacement of muscle potassium by sodium in normal dogs. Am. J. Physiol., 1941, **135**, 230.
11. Darrow, D. C., and Miller, H. C., The production of cardiac lesions by repeated injections of desoxycorticosterone acetate. J. Clin. Invest., 1942, **21**, 601.
12. Davis, A. K., Bass, A. C., and Overman, R. R., Comparative effects of cortisone and DCA on ionic balance and fluid volumes of normal and adrenalectomized dogs. Am. J. Physiol., 1951, **166**, 493.
13. Tobian, L., Jr., and Binion, J., Artery wall electrolytes in renal and DCA hypertension. J. Clin. Invest., 1954, **33**, 1407.
14. Ziegler, M., Anderson, J. A., and McQuarrie, I., Effects of desoxycorticosterone acetate on water and electrolyte content of brain and other tissues. Proc. Soc. Exper. Biol. & Med., 1944, **56**, 242.
15. Laramore, D. C., and Grollman, A., Water and electrolyte content of tissues in normal and hypertensive rats. Am. J. Physiol., 1950, **161**, 278.
16. Greene, R. W., and Sapirstein, L. A., Total body sodium, potassium and nitrogen in rats made hypertensive by subtotal nephrectomy. Am. J. Physiol., 1952, **169**, 343.
17. Ross, E. J., Total exchangeable sodium in hypertensive patients. Clin. Sc., 1956, **15**, 81.
18. Tobian, L., and Binion, J. T., Tissue cations and water in arterial hypertension. Circulation, 1952, **5**, 754.
19. Eichelberger, L., The distribution of water and electrolytes between blood and skeletal muscle in experimental hypertension. J. Exper. Med., 1943, **77**, 205.
20. Ledingham, J. M., The distribution of water, sodium, and potassium in heart and skeletal muscle in experimental renal hypertension in rats. Clin. Sc., 1953, **12**, 337.
21. Grollman, A., The water and electrolyte content of the tissues in hypertension. Circ. Research, 1954, **2**, 541.
22. Harrison, H. E., and Darrow, D. C., The distribution of body water and electrolytes in adrenal insufficiency. J. Clin. Invest., 1938, **17**, 77.
23. Muntwyler, E., Mellors, R. C., Mautz, F. R., and Mangun, G. H., Electrolyte and water equilibria in the dog. II. Electrolyte and water exchange between skeletal muscle and blood in adrenal insufficiency. J. Biol. Chem., 1940, **134**, 367.

SPECIAL NOTICE TO SUBSCRIBERS

Post Offices will no longer forward the Journal when you move.

Please notify The Journal of Clinical Investigation, Business Office, 333 Cedar Street, New Haven 11, Conn. at once when you have a change of address, and do not omit the zone number if there is one.

THE PHYSIOLOGICAL DISPOSITION AND METABOLIC FATE OF CORTISONE IN MAN

By RALPH E. PETERSON, CHARLES E. PIERCE, JAMES B. WYNGAARDEN,[1] JOSEPH J. BUNIM, AND BERNARD B. BRODIE

(From the National Institute of Arthritis and Metabolic Diseases, and the National Heart Institute, National Institutes of Health, Bethesda, Md.)

(Submitted for publication March 15, 1957; accepted May 2, 1957)

In an earlier paper we described studies on the physiological disposition and metabolic fate of hydrocortisone in man (1). This paper describes similar studies on cortisone. Although contributing very little to the body pool of adrenal cortical steroids, cortisone has been used extensively as a therapeutic agent, and it was therefore of interest to compare its metabolism with that of hydrocortisone. Large doses of cortisone and hydrocortisone were administered intravenously and rates of disappearance from the plasma determined in normal subjects and in patients with liver disease, myxedema, thyrotoxicosis, and rheumatoid arthritis. Trace quantities of cortisone-4-C^{14} and hydrocortisone-4-C^{14} were employed in certain studies. From these studies, information was obtained regarding rate of metabolic transformation, rate of absorption, rates and routes of excretion of cortisone and its metabolites, and, to some extent, the identity and amount of these metabolites. Information was also obtained on the *in vivo* conversion of cortisone to hydrocortisone.

MATERIALS AND METHODS

Administration of steroid. The steroids were dissolved in 25 ml. ethanol and added to 500 ml. of sterile five per cent dextrose in water. This solution was administered intravenously over a period of 10 to 20 minutes. For oral or intramuscular administration the steroids were suspended in saline. Cortisone-4-C^{14} was administered intravenously in trace quantities in a small volume of five per cent ethanol in water over a period of 3 to 5 minutes, or mixed with carrier cortisone and given in 500 ml. of three per cent ethanol in five per cent dextrose in sterile distilled water. Following the administration of the steroids, blood samples were drawn in heparinized syringes every 20 or 30 minutes for 2 hours or more. Urine samples were collected and preserved by freezing.

Methods of assay. The extraction procedures for the steroids in plasma and urine were the same as those described previously (1). Cortisone and certain of its metabolites in plasma were determined by a modification (2) of the original phenylhydrazine procedure of Silber and Porter (3). Specificity of this method of assay for plasma hydrocortisone after administration of hydrocortisone was shown to be high, as tested by counter-current distribution and isotope dilution techniques. Using these same techniques, it was found that the Silber-Porter method was much less satisfactory for the specific measurement of cortisone after the administration of cortisone.

Counter-current distribution. Plasma drawn 60 minutes after a 20-minute intravenous infusion of 200 mg. of cortisone was extracted with dichloromethane. A trace quantity of cortisone-4-C^{14} was added to the dichloromethane extract. The extract was evaporated to dryness, and the apparent cortisone subjected to a 16-transfer counter-current distribution. The solvents used were one volume of water as the upper phase and one volume of a mixture containing four parts carbon tetrachloride and one part dichloromethane as the lower phase. This system gave a partition coefficient of 1.5 for cortisone. After distribution, aliquots of both phases were assayed by the phenylhydrazine reaction, and for carbon^{14}. Figure 1 shows the distribution of the phenylhydrazine reacting material in plasma, and the cortisone-4-C^{14}. The data indicate that more than one-half of the dichloromethane-soluble material in plasma reacting with phenylhydrazine was not cortisone. The major metabolites giving the phenylhydrazine reaction are distributed in a manner like hydrocortisone ($K = 5.2$) and tetrahydrocortisone ($K = 4.8$).

Isotope dilution. The plasma samples obtained following the administration of cortisone were subjected to an isotope dilution assay, and additional aliquots were assayed by the phenylhydrazine procedure. The isotope dilution assay was carried out by first adding a known quantity of cortisone-4-C^{14} to a known volume of plasma. The plasma was extracted with dichloromethane, and the dichloromethane extract evaporated to dryness *in vacuo* and subjected to paper chromatography in a modified Bush-type (4) system (cyclohexane 2: benzene 4: methanol 4: water 1), along with a cortisone standard. Following development of the chromatogram for 18 to 24 hours, the paper strip was removed and scanned with ultraviolet light. The cortisone reference standard was located and marked (cortisone moves about 20 cm. in 24 hours). The band of paper containing the plasma

[1] Present address: Department of Medicine, Duke University, Durham, North Carolina.

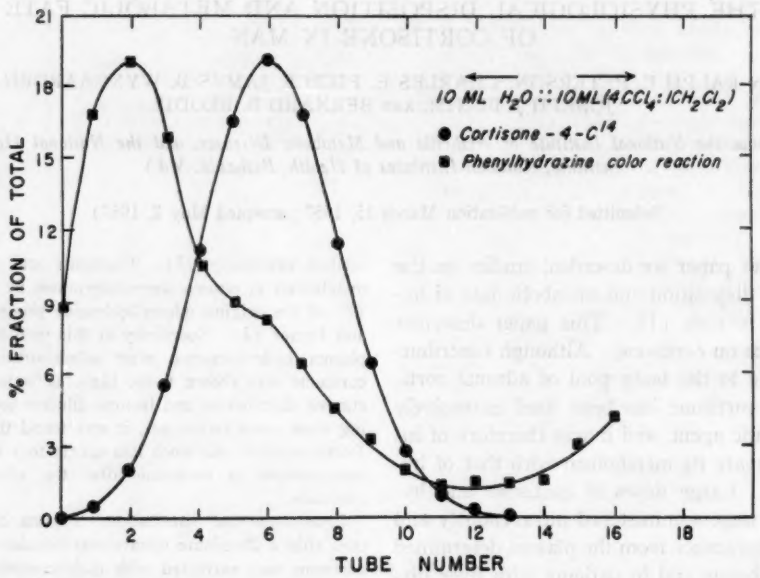


FIG. 1. COUNTER-CURRENT DISTRIBUTION OF PLASMA STEROIDS REACTING WITH PHENYLHYDRAZINE 60 MINUTES AFTER INFUSION OF 200 MG. CORTISONE

cortisone was cut into a very narrow strip, perpendicular to the running direction of the solvent. The cortisone was eluted from this paper strip with 3 ml. of 95 per cent cold ethanol and the ethanol evaporated to dryness under a stream of nitrogen.

The residue was dissolved in a small volume of dichloromethane (4 to 8 ml.). One aliquot was taken for phenylhydrazine assay, and another for carbon^{14} assay. The specific activity of the cortisone expressed in counts per minute per microgram was then determined from the radioactivity value and the quantitative cortisone value as determined by the phenylhydrazine assay. The quantity of cortisone in the plasma sample was calculated as previously described (2).

Procedure for determination of cortisone metabolites in urine. The method and reagents were the same as those previously described for hydrocortisone (1).

Studies with cortisone-4-C^{14}. Cortisone-4-C^{14} acetate (0.49 mc. per mM), hydrocortisone-4-C^{14} (1.47 mc. per mM), tetrahydrocortisone-4-C^{14} mono-acetate (0.4 mc. per mM), and dihydrocortisone-4-C^{14} acetate (0.4 mc. per mM) were made available through the Endocrine Study Section of the National Institutes of Health.[2] The acetates were converted to the free steroids by hydroylsis at pH 7.4 with acetyl cholinesterase (Nutritional Bio-Chemicals Corp.), 500 units per 3 ml. 0.5 M glycyl glycine buffer. The buffered solution was incubated at 37° C. for 18 hours, and then was extracted with dichloromethane. The dichloromethane was evaporated to dryness under nitrogen, and the residue chromatographed on paper for 18 hours (cyclohexane 2: benzene 4: methanol 4: water 1). The free cortisone, tetrahydrocortisone, and dihydrocortisone were eluted from the paper with 95 per cent ethanol. Reverse isotope dilution analysis, using paper chromatography, showed these to be essentially pure steroids.

The plasma and urine samples for radioactivity assay were extracted in the same manner as for the colorimetric assays; however, the dichloromethane extracts were transferred to planchets for counting.

The studies on the biliary excretion of the radiometabolites of cortisone-4-C^{14} were made on the same patient who was used for the hydrocortisone-4-C^{14} studies (1).

Procedure for carbon^{14} analysis of samples. All assays for carbon^{14} were carried out with a Robinson (6) gas-flow counter as previously described (1). Plasma, urine, and bile samples were counted as previously described (1).

RESULTS

Rate of disappearance of cortisone and "cortisone-like" material from plasma after intravenous administration

When the concentrations of phenylhydrazine-reacting material present in the plasma after the infusion of cortisone were plotted against time on

[2] Carbon^{14}-labeled tetrahydrohydrocortisone and dihydrohydrocortisone were prepared enzymatically from hydrocortisone-4-C^{14} (5).

semilogarithmic paper, a straight line was obtained. (In a few of the subjects with cirrhosis, *e.g.*, Figure 5, a single straight line was not observed.) In normal subjects the half-time of disappearance of the material reacting with phenylhydrazine was the same, irrespective of the quantity of cortisone administered over a range of 50 to 400 mg. This indicated that the rate of disappearance of cortisone (and its metabolites) was proportional to concentration over a wide range of concentrations. This permitted a comparison of the behavior of infused cortisone in different subjects under various conditions.

In 15 normal subjects, the mean biological half-time of the material reacting with phenylhydrazine was 60 minutes, with a range of 45 to 95 minutes (Figure 2). In patients with cirrhosis of the liver and myxedema, the biological half-times were not significantly prolonged. In the subjects with thyrotoxicosis, the half-times were shortened (mean, 28 minutes) and in the rheumatoid arthritis patients, the half-times were within the normal range.

In all groups except for the subjects with thyrotoxicosis, the differences in the half-times for the material reacting with phenylhydrazine were not greatly different from those in the normals, and thus it was not possible to be sure that cortisone *per se* was being metabolized at a different rate. Since both counter-current distribution and isotope dilution analysis of the material reacting with phenylhydrazine indicated that a large fraction of the steroid measured with the phenylhydrazine reagent was not cortisone, it was of interest to determine the rate of disappearance of *true cortisone* in these groups (Table I). In four normal subjects, cortisone, as determined by isotope dilution assay, was found to disappear at a rate approximately twice that of the material reacting with phenylhydrazine. In three patients with cirrhosis of the liver and in one patient with myxedema, cortisone was also found to disappear at the normal rate. However, in one patient with thyrotoxicosis, the half-time for true cortisone (12 minutes) and the steroids reacting with phenylhydrazine (30 minutes) were both much reduced. In all cases the actual cortisone disappeared at approximately twice the rate of the phenylhydrazine-reacting material.

TABLE I

Disappearance of cortisone and phenylhydrazine-reacting steroids from plasma following intravenous administration of cortisone

	Biological half-times (*minutes*)	
	Phenyl-hydrazine	Cortisone
Normal	55	23
Normal	50	30
Normal	60	35
Normal	55	25
Cirrhosis	60	30
Cirrhosis	70	30
Cirrhosis	60	30
Myxedema	60	25
Thyrotoxicosis	30	12

Disappearance of radioactive cortisone from plasma

Following intravenous infusion of cortisone-4-C^{14}, the concentration in plasma of isotopic substances extractable with dichloromethane decreased

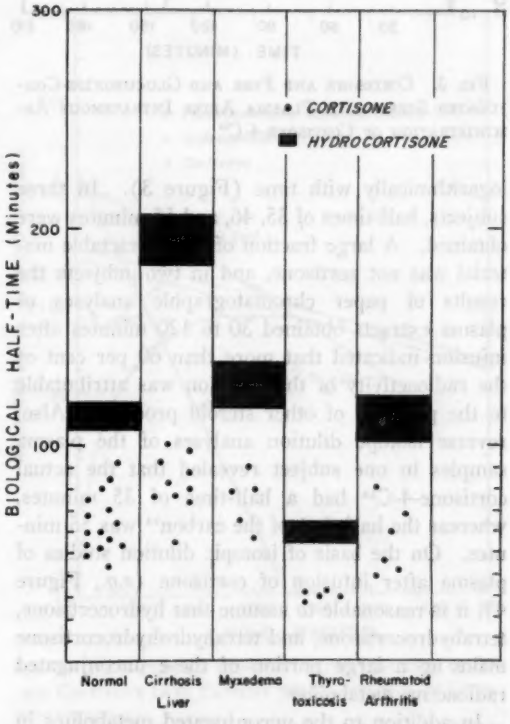


FIG. 2. BIOLOGICAL HALF-TIMES OF STEROIDS REACTING WITH PHENYLHYDRAZINE FOLLOWING INTRAVENOUS ADMINISTRATION OF HYDROCORTISONE AND CORTISONE

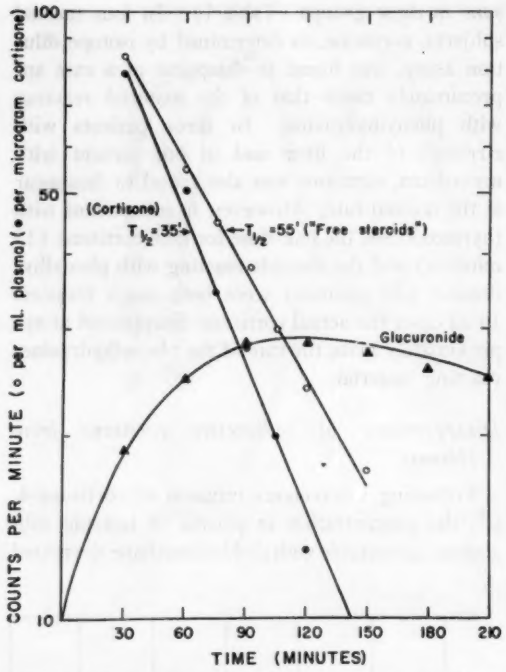


FIG. 3. CORTISONE AND FREE AND GLUCURONIDE-CONJUGATED STEROIDS IN PLASMA AFTER INTRAVENOUS ADMINISTRATION OF CORTISONE-4-C^{14}

logarithmically with time (Figure 3). In three subjects, half-times of 35, 46, and 55 minutes were obtained. A large fraction of the extractable material was not cortisone, and in two subjects the results of paper chromatographic analyses of plasma extracts obtained 30 to 120 minutes after infusion indicated that more than 60 per cent of the radioactivity of this fraction was attributable to the presence of other steroid products. Also, reverse isotope dilution analyses of the plasma samples in one subject revealed that the actual cortisone-4-C^{14} had a half-time of 35 minutes, whereas the half-time of the carbon^{14} was 55 minutes. On the basis of isotopic dilution studies of plasma after infusion of cortisone (*e.g.*, Figure 4), it is reasonable to assume that hydrocortisone, tetrahydrocortisone, and tetrahydrohydrocortisone make up a large portion of these unconjugated radioactive metabolites.

In addition to the unconjugated metabolites in plasma, there was present a fraction of labeled metabolites that was rendered dichloromethane soluble following β-glucuronidase hydrolysis. As the free steroids disappeared from plasma, conjugated derivatives hydrolyzable with β-glucuronidase appeared, and reached a maximum at 90 to 120 minutes. At 90 minutes the free and conjugated fractions were of equal magnitude. Thereafter, the conjugated fraction exceeded the level of the free, and disappeared from the plasma at a rate slower than that of the free fractions.

Comparative rates of disappearance from plasma of steroids structurally related to cortisone

Table II shows the rates of disappearance from the plasma of several steroids structurally similar to cortisone, in a normal subject and in one patient with cirrhosis of the liver. The phenylhydrazine method was used for assay of all of these steroids except cortisone, and with all the steroids, except for cortisone, the assay method has been found to be relatively specific (on the basis of evaluation by isotope dilution assay). In the normal subject and the patient with cirrhosis, cortisone and the dihydro and tetrahydro derivatives of cortisone and hydrocortisone disappear much more rapidly than hydrocortisone.

In vivo conversion of cortisone to hydrocortisone

Cortisone was infused over a period of 12 minutes into a normal subject and a patient with cirrhosis of the liver, and blood samples were collected at various times after the termination of the infusion. Endogenous adrenal hydrocortisone production was suppressed by the prior administration of Δ^1,9α-fluorohydrocortisone (2.5 mg. per day). The plasma samples were assayed for total

TABLE II

Biological half-times of various steroids following their intravenous administration

	Biological half-times (*minutes*)	
	Normal	Cirrhosis
Hydrocortisone	98	320
Cortisone*	23	30
Dihydrocortisone	25	32
Tetrahydrocortisone	27	25
Dihydrohydrocortisone	25	25
Tetrahydrohydrocortisone	23	27

* Cortisone was determined by the isotope dilution procedure, and all other steroids by the phenylhydrazine method.

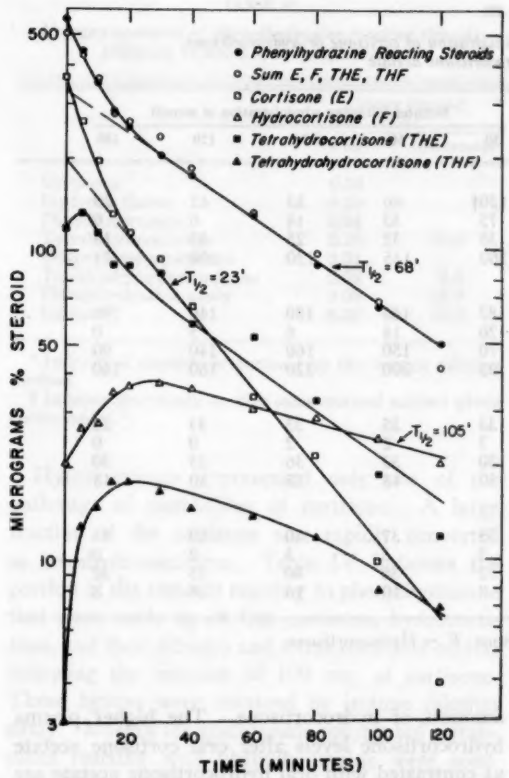


FIG. 4. FREE PLASMA STEROIDS AFTER INFUSION OF 400 MG. CORTISONE INTO NORMAL SUBJECT

material reacting with phenylhydrazine. With the use of the isotope dilution procedure with paper chromatography, the samples were assayed for hydrocortisone, cortisone, tetrahydrocortisone, and tetrahydrohydrocortisone.[3] In the normal subject, one-half of the cortisone had disappeared in 23 minutes. Within one hour, the concentration of hydrocortisone and of tetrahydrocortisone equaled the level of cortisone, and after two hours the cortisone could no longer be detected (Figure 4). Figure 5 shows the results obtained in the patient with cirrhosis of the liver. Cortisone disappeared at a normal rate, and after two hours the level of the material reacting with phenylhydrazine approximated the level of free hydrocortisone.

Three hundred mg. of carrier cortisone plus 3 microcuries of cortisone-4-C^{14} were infused into a normal subject over a period of 10 minutes. Endogenous hydrocortisone production was suppressed by the prior administration of Δ^1,9α-fluorohydrocortisone. Blood samples were collected every 30 minutes during the two-hour period following termination of the infusion. Each plasma sample was extracted with dichloromethane and subjected to paper chromatography, and the cortisone and hydrocortisone eluted from the paper with 95 per cent ethanol. The specific activity of each steroid was determined. All samples of cortisone and hydrocortisone gave similar (± 10 per cent) specific activities. With labeled hydrocortisone, it was not possible to detect any conversion of hydrocortisone to cortisone.[4]

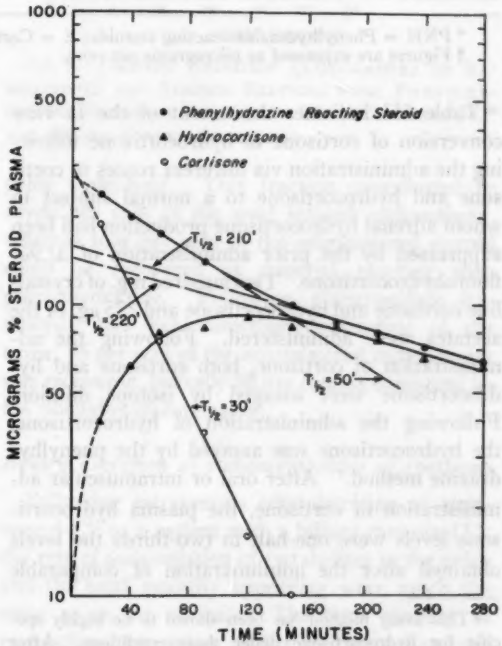


FIG. 5. FREE PLASMA STEROIDS AFTER INFUSION OF 200 MG. CORTISONE INTO PATIENT WITH CIRRHOSIS OF LIVER

[3] The accuracy of the isotope dilution assay was not in every instance checked by purification of the samples to a constant specific activity. In many assays, however, the samples were either re-chromatographed in different solvent systems or converted to their respective acetates and re-chromatographed. Whenever these additional purifications were carried out, the repeat specific activities checked with the first assay to within the average error of the method ± 10 per cent.

[4] Some conversion of hydrocortisone to cortisone was shown to occur, however, as was evident from the isolation of C^{14}-labeled cortisone from the urine, after administration of C^{14}-labeled hydrocortisone.

TABLE III

Plasma steroid concentration following administration of cortisone or hydrocortisone, and cortisone or hydrocortisone acetate

	Minutes following administration of steroid				
	30	60	90	120	180
Cortisone, 200 mg. intravenously					
PNH*	120†	86	53	42	20
E	75	33	14	6	0
F	38	32	25	32	14
Hydrocortisone, 200 mg. intravenously	160	135	120	100	71
Cortisone, 200 mg. orally					
PNH	87	160	180	140	96
E	20	14	6	3	0
F	70	150	160	140	90
Hydrocortisone, 200 mg. orally	95	200	220	180	140
Cortisone, 200 mg. intramuscularly					
PNH	33	35	35	33	30
E	3	2	2	0	0
F	30	33	36	35	30
Hydrocortisone, 200 mg. intramuscularly	30	48	55	50	45
Cortisone acetate, 225 mg. orally					
PNH	35	37	40	40	35
E	2	3	3	2	0
F	32	36	40	35	32
Hydrocortisone acetate, 225 mg. orally	8	10	16	16	8

* PNH = Phenylhydrazine-reacting steroids; E = Cortisone; F = Hydrocortisone.
† Figures are expressed as micrograms per cent.

Table III indicates the extent of the *in vivo* conversion of cortisone to hydrocortisone following the administration via different routes of cortisone and hydrocortisone to a normal subject in whom adrenal hydrocortisone production had been suppressed by the prior administration of Δ^1,9α-fluorohydrocortisone. Two hundred mg. of crystalline cortisone and hydrocortisone and 225 μg. of the acetates were administered. Following the administration of cortisone, both cortisone and hydrocortisone were assayed by isotope dilution. Following the administration of hydrocortisone, the hydrocortisone was assayed by the phenylhydrazine method.[5] After oral or intramuscular administration of cortisone, the plasma hydrocortisone levels were one-half to two-thirds the levels obtained after the administration of comparable amounts of hydrocortisone. The higher plasma hydrocortisone levels after oral cortisone acetate as contrasted with oral hydrocortisone acetate are the result of the incomplete absorption of hydrocortisone acetate (unpublished observations). Following intravenous administration of cortisone, lower hydrocortisone levels are obtained, presumably because via this route of administration a larger fraction of the cortisone is rapidly transformed to tetrahydrocortisone.

Excretion of cortisone and metabolites

Following intravenous administration of cortisone, 30 to 40 per cent of the administered steroid was recovered as material reacting with phenylhydrazine in the dichloromethane extract of glucuronidase-treated urine during the first 24 hours. Approximately one-half of the recovered material appeared in the urine during the first four hours. Direct extraction of the urine with dichloromethane yielded phenylhydrazine substances equivalent to 3 to 7 per cent of the administered dose, and most of these unconjugated steroids were excreted during the first six hours.

[5] This assay method has been shown to be highly specific for hydrocortisone under these conditions. After oral or intramuscular administration of hydrocortisone or hydrocortisone acetate, more than 90 per cent of the steroid reacting with phenylhydrazine was hydrocortisone, and after intravenous administration of hydrocortisone, 80 to 90 per cent of the steroid reacting with phenylhydrazine was found to be hydrocortisone.

TABLE IV

Urinary excretion of phenylhydrazine-reacting steroids following infusion of 100 mg. cortisone

	Urinary steroids* (%)	
	Free	Glucuronide
Cortisone	0.30	
Hydrocortisone	0.60	
Dihydrocortisone	0.04	
Tetrahydrocortisone	0.20	20.0
Dihydrohydrocortisone	0.01	
Tetrahydrohydrocortisone	0.08	6.0
Phenylhydrazine assay	3.00	28.0
Carbon^{14}†	6.00	52.0

* Individual steroids determined by the isotope dilution method.

† Independent study on this same normal subject given cortisone-4-C^{14}.

Hydrocortisone represented only one of the pathways of metabolism of cortisone. A large fraction of the cortisone was rapidly converted to tetrahydrocortisone. Table IV indicates the portion of the steroids reacting to phenylhydrazine that were made up of free cortisone, hydrocortisone, and their dihydro and tetrahydro derivatives, following the infusion of 100 mg. of cortisone. These figures were obtained by isotope dilution assay. Most of the glucuronide conjugated metabolites reacting with phenylhydrazine were compounds more polar than cortisone, and more than seventy per cent of the steroid glucuronides reacting with phenylhydrazine consisted of tetrahydrocortisone.

Urinary excretion of radiometabolites of cortisone

Figure 6 shows the cumulative urinary excretion of labeled cortisone and its metabolites following intravenous administration of a tracer quantity of cortisone-4-C^{14} plus 200 mg. carrier cortisone. Eighty per cent of the injected dose was excreted within the first 24 hours. A small additional quantity appeared during the second, and a trace during the third 24-hour period. By the fourth day, no labeled urinary products could be demonstrated. In three such studies, an average of 91 per cent of the infused radioactivity was accounted for through urinary excretion. The half-time of the initial excretion rate of these products averaged 3.6 hours. During the first six hours, there was excreted a urinary fraction which was extractable with dichloromethane without

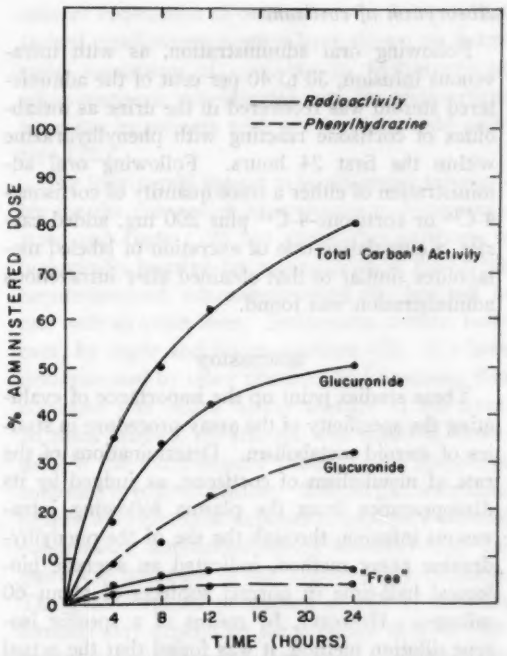


FIG. 6. URINARY EXCRETION (CUMULATIVE) OF RADIOACTIVITY AND STEROIDS REACTING WITH PHENYLHYDRAZINE FOLLOWING INFUSION OF 1.1 μC. CORTISONE-4-C^{14} AND 200 MG. CARRIER CORTISONE

prior hydrolysis. This fraction contributed to seven per cent of the total infused radioactivity, whereas four per cent of the administered steroid could be recovered as material reacting with phenylhydrazine. Following glucuronidase hydrolysis and dichloromethane extraction of the urine, 55 per cent of the administered radioactivity was recovered, but only 30 per cent as measured by the phenylhydrazine assay.

Biliary excretion of radiometabolites of cortisone

Following intravenous administration of cortisone-4-C^{14} to a patient with a biliary cannula (1), the rate of accumulation of metabolites in the urine and the total quantity appearing were much the same as in the normals. The total quantity of metabolites appearing in the bile was four per cent of the injected dose. Analysis of the feces collected for three days following the infusion of the steroid failed to yield any radioactivity. Direct dichloromethane extraction of the bile failed to reveal the presence of any radioactivity.

Absorption of cortisone

Following oral administration, as with intravenous infusion, 30 to 40 per cent of the administered steroid was recovered in the urine as metabolites of cortisone reacting with phenylhydrazine within the first 24 hours. Following oral administration of either a trace quantity of cortisone-4-C^{14} or cortisone-4-C^{14} plus 200 mg. added carrier, a cumulative rate of excretion of labeled metabolites similar to that obtained after intravenous administration was found.

DISCUSSION

These studies point up the importance of evaluating the specificity of the assay procedure in studies of steroid metabolism. Determinations of the rate of metabolism of cortisone, as judged by its disappearance from the plasma following intravenous infusion, through the use of the phenylhydrazine assay method, indicated an average biological half-time in normal subjects of about 60 minutes. However, by means of a specific isotope dilution method, it was found that the actual cortisone had an average plasma biological half-time of 28 minutes. It was shown that the phenylhydrazine method measured the combined rates of disappearance of cortisone and metabolites of cortisone (hydrocortisone, tetrahydrohydrocortisone, and tetrahydrocortisone). The disappearance of true cortisone, as determined by an isotope dilution assay, was found in all cases to have the characteristics of a first-order reaction. Since less than one per cent of the administered cortisone was excreted as unaltered steroid, the rate of disappearance of cortisone from plasma was presumably a measure of its rate of metabolism.

In patients with liver disease, cortisone disappeared from the plasma at the normal rate, whereas hydrocortisone has been shown to be metabolized at a much slower rate (1, 7). In patients with liver disease, cortisone was metabolized at the same rapid rate as the biologically inactive dihydro and tetrahydro metabolites of cortisone and hydrocortisone.

Patients with thyrotoxicosis showed an increased rate of metabolism of both cortisone and hydrocortisone. Corticosterone has also been found to be metabolized at an increased rate in thyrotoxicosis (8). This may indicate that in thyrotoxicosis there is a general increased turnover of the various steroid dihydro-dehydrogenase enzymes required for saturation of ring A of many of the steroids.

These data on the metabolism of cortisone are at variance with those of Eik-Nes, Sandberg, Tyler, and Samuels (9), who, using a phenylhydrazine assay, reported that cortisone and hydrocortisone were metabolized at the same rate in normal subjects.

Cortisone was metabolized at a rate approximately four times faster than that of hydrocortisone; however, cortisone has approximately two-thirds the biological activity of hydrocortisone. This indicates a clear lack of correlation between rate of metabolism of a steroid known to be biologically potent and its biological activity as observed in man and animals.[6] Since, however, such a large fraction of the administered cortisone was found to be rapidly converted to hydrocortisone, it may be that most of the biological activity of cortisone resides in that fraction that is converted to hydrocortisone.

Previous investigators have shown by perfusion of the liver (11, 12) and with liver slices (13, 14) and liver homogenates (15, 16), that cortisone is converted to hydrocortisone. Also, studies of urinary steroid metabolites in man following the administration of cortisone have demonstrated this conversion (17–19). Attempts to evaluate the extent of the conversion of cortisone to hydrocortisone through studies of the levels of cortisone and hydrocortisone in the urine may give an incomplete picture, since only a small fraction of the administered steroid is excreted as cortisone and hydrocortisone. Studies of plasma levels give a better indication of the initial sequence of metabolic transformations of administered steroid.

Our studies demonstrated that a large fraction of the administered cortisone was converted to hydrocortisone, and the relative ratio of hydrocortisone to cortisone in the plasma after administration of cortisone was very much dependent on the method of administration. Following a rapid infusion of a large amount of cortisone, a large fraction of the cortisone was converted initially to

[6] 9α-fluorohydrocortisone, a steroid possessing considerably more biological activity than hydrocortisone, has been found to be metabolized at a slightly more rapid rate than hydrocortisone (unpublished observations [10]).

tetrahydrocortisone. After the oral or intramuscular administration of cortisone, hydrocortisone made up the largest fraction of phenylhydrazine steroid in the plasma, and following orally administered cortisone acetate with its slower absorption, hydrocortisone accounted for 90 to 95 per cent of the plasma steroid reacting with phenylhydrazine. Thus, with the usual routes of administration of cortisone as it is used therapeutically, the plasma levels of hydrocortisone were one-half to two-thirds those obtained after administration of comparable amounts of hydrocortisone. This difference cannot be explained on the basis of a more incomplete absorption of the orally administered cortisone (Figure 6). Cortisone acetate, like cortisone and hydrocortisone, has been found to be completely absorbed after oral administration, but at a slightly slower rate. The lower plasma hydrocortisone levels after orally administered hydrocortisone acetate are the result of its incomplete absorption.

Clinical studies on the relative potency of cortisone versus hydrocortisone administered orally to humans have shown that, on a weight basis, cortisone is approximately two-thirds as effective as hydrocortisone as an anti-inflammatory agent (20, 21). Cortisone administered orally also appears to be about two-thirds as effective as hydrocortisone in suppression of the adrenal (22). In addition, studies on the relative potency of cortisone and hydrocortisone in the rat, through the use of such indices as thymic involution, liver glycogen deposition, and the muscle work test, have shown that cortisone is only about two-thirds as active as hydrocortisone (23). Thus, by several criteria, cortisone has been shown to be biologically less active than hydrocortisone. From our observations it would appear that one could account for most, if not all, of the biological activity of cortisone on the basis of that fraction that is converted to hydrocortisone (plasma levels of hydrocortisone after cortisone, one-half to two-thirds the levels of hydrocortisone after comparable doses of hydrocortisone). Intra-articularly injected cortisone or its acetate, though reported to be converted in part to hydrocortisone (24), has been found to be very much less effective in the relief of joint symptoms than either hydrocortisone or its acetate (25–27). This lack of effectiveness may be due to the failure of the synovium to effect any significant conversion of cortisone to hydrocortisone. Indeed, preliminary studies have shown no detectable conversion of cortisone to hydrocortisone when cortisone was injected into the intra-articular space in patients with active rheumatoid arthritis (28).

The liver would appear to be the organ responsible for the conversion of cortisone to hydrocortisone, and Ingle (29) has stated that cortisone is biologically inactive for maintenance of life in the hepatectomized, adrenalectomized rat, but not in rats with an intact liver. Subsequent studies, however, by Ingle and his co-workers (30, 31) have demonstrated by other physiological functions that cortisone was active in the absence of the liver. In these studies, no attempt was made to determine if the extra-hepatic tissues could transform cortisone to hydrocortisone. Steroid glucocorticoid properties (glycogen deposition, pituitary-adrenal suppression, anti-inflammatory activity) may require the presence of an 11β-hydroxy function. The glucocorticoid properties of the 11-dehydro steroids may depend on the presence of liver 11β-hydroxy dehydrogenase enzymes. Preliminary unpublished data on the metabolism of Δ^1-cortisone have shown that following oral administration of this steroid, the plasma levels of Δ^1-hydrocortisone far exceed the Δ^1-cortisone levels. This again suggests that the biological activity of the administered 11-dehydro compound (Δ^1-cortisone) may reside in that fraction that is transformed to Δ^1-hydrocortisone. It is also of interest to note that the 2-methyl analogues of cortisone and 9α-fluorohydrocortisone have very much less glucocorticoid activity than the parent 11β-hydroxy steroids (32). It will be of interest to determine if the liver *in vivo* is unable to effect a reduction of the 11-keto groups of the 2-methyl steroids.

The major route of excretion of labeled metabolites of cortisone was via the urine, in which up to 91 per cent of the administered carbon^{14} appeared in 72 hours. Only a minor fraction of the steroid (about four per cent) was excreted into the gastrointestinal tract via the bile. Previous results obtained with hydrocortisone were similar (1, 33, 34). Studies with corticosterone have yielded similar results; however, slightly more is excreted through the bile and less via the urine (8, 35).

In the present study, recoveries of metabolites in urine indicated that three to seven per cent of the

cortisone, when administered either orally or intravenously, was directly extractable with dichloromethane without prior hydrolysis. All of these freely extractable steroids appeared within the first six to eight hours after administration. Only 0.3 per cent of the administered steroid was excreted as unaltered cortisone, and the remaining steroids consisted for the most part of hydrocortisone, tetrahydrocortisone, and tetrahydrohydrocortisone.

Urinary metabolites equivalent to 50 to 60 per cent of the administered cortisone-4-C^{14} were released by enzymatic hydrolysis (β-glucuronidase) as determined by radioactivity analysis. Thirty to 40 per cent of the administered cortisone was accounted for in the urine as metabolites reacting with phenylhydrazine.

These data agree well with previously published reports on cortisone metabolites extractable with butanol (36), or extractable with chloroform or similar solvents following β-glucuronidase hydrolysis (37, 38). The disparity between the carbon^{14} and phenylhydrazine analyses may be in large measure accounted for in the same way as with the hydrocortisone metabolites (39)—the presence of C_{20}-hydroxy compounds that do not react with phenylhydrazine. Also, a small fraction of the non-phenylhydrazine reacting material may be C_{19}-17 ketosteroids. Tetrahydrocortisone made up the major portion of the metabolites reacting with phenylhydrazine, and tetrahydrocortisone and tetrahydrohydrocortisone accounted for 93 per cent of all the phenylhydrazine-reactive glucuronide conjugates.[7]

After prior hydrolysis of the conjugated products with β-glucuronidase and extraction of released steroids with dichloromethane, an additional 5 to 10 per cent of the infused isotope can be extracted with ethyl acetate, and another 10 to 20 per cent with n-butanol saturated with water. Whether these fractions represent glucuronides hydrolyzable but not extracted with dichloromethane, glucuronides resisting hydrolysis with bacterial enzymes, or sulfates or other conjugation products, remains to be determined, as does the nature of the metabolites in the urine residue completely resisting extraction (five to ten per cent). Acid hydrolysis at room temperature for 24 hours will not make this latter fraction soluble in organic solvents.

[7] The following unpublished data may be pertinent to the findings that following intravenous administration of cortisone, the urinary concentration of tetrahydrocortisone glucuronide greatly exceeds the concentration of tetrahydrohydrocortisone glucuronide: Following intravenous infusion of tetrahydrocortisone, seven per cent was recovered in the urine as tetrahydrohydrocortisone, and 40 per cent as tetrahydrocortisone after glucuronidase hydrolysis. Twenty per cent of infused tetrahydrohydrocortisone was recovered in the urine as tetrahydrocortisone, and 30 per cent as tetrahydrohydrocortisone after glucuronidase hydrolysis. Thus, the presence of the α, β unsaturated 3-keto group in ring A seems to direct the enzymatic reduction of cortisone → hydrocortisone, whereas the saturated ring A seems to favor oxidation of the 11β-hydroxy group (tetrahydrohydrocortisone → tetrahydrocortisone).

SUMMARY

The physiological disposition and metabolic fate of cortisone were studied in man following intravenous infusion of steroid in doses up to 400 mg. In normal subjects, the biological half-life of the steroids reacting with phenylhydrazine in the plasma after infusion of cortisone was shown to average 60 minutes. Following infusion of tracer quantities of cortisone-4-C^{14}, the biological half-life of the unconjugated radioactive metabolites was found to be 35 to 55 minutes. A large fraction of these steroids measured with the phenylhydrazine reagent and by radioactive assay was, however, not cortisone. The biological half-life of the actual cortisone was found by a specific isotope dilution assay procedure to average 28 minutes in normal subjects. In patients with thyrotoxicosis, cortisone was found to disappear from the plasma at an increased rate, and in patients with liver disease, cortisone disappeared at the normal rate.

Administered cortisone was found to be very rapidly metabolized to hydrocortisone. The plasma hydrocortisone levels after oral or intramuscular administration of cortisone were found to be one-half to two-thirds those found following administration of comparable amounts of hydrocortisone. The absorption of cortisone from the gastrointestinal tract was found to be both rapid and complete. It was suggested that most, if not all, of the biological activity of cortisone could be accounted for by that fraction that was transformed to hydrocortisone.

Normal subjects excreted approximately 90 per cent of the administered steroid as urinary metabo-

lites in 72 hours, as judged from excretion of carbon[14] following infusion of tracer or larger quantities of labeled steroid. Four per cent was excreted in the bile. Three to seven per cent was excreted in the urine as unconjugated substances; 0.3 per cent as unchanged cortisone, and another 0.6 per cent as hydrocortisone. About 55 per cent of the administered steroid was excreted as metabolites hydrolyzable with bacterial β-glucuronidase; however, only about one-half of this fraction was detectable with the phenylhydrazine reaction, and tetrahydrocortisone was found to make up 70 per cent of this latter fraction.

ACKNOWLEDGMENT

We are indebted to the technical assistance of Serafim L. Guerra, Miss Aurora Karrer, and Miss Margaret Bollier. The generous supplies of steroid were kindly supplied by Merck and Company and by the Upjohn Company.

REFERENCES

1. Peterson, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B., and Bunim, J. J., The physiological disposition and metabolic fate of hydrocortisone in man. J. Clin. Invest., 1955, **34**, 1779.
2. Peterson, R. E., Karrer, A., and Guerra, S. L., Evaluation of the Silber-Porter procedure for determination of plasma hydrocortisone. Anal. Chem., 1957, **29**, 144.
3. Silber, R. H., and Porter, C. C., The determination of 17,21-dihydroxy-20-ketosteroids in urine and plasma. J. Biol. Chem., 1954, **210**, 923.
4. Bush, I. E., Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. Biochem. J., 1952, **50**, 370.
5. Tomkins, G. M., Enzymatic mechanisms of hormone metabolism. I. Oxidation-reduction of the steroid nucleus. Recent Progress in Hormone Research, vol. 12. New York, Academic Press, 1956, p. 125.
6. Robinson, C. V., Windowless, flow-type proportional counter for counting C^{14}. Science, 1950, **112**, 198.
7. Brown, H., Willardson, D. G., Samuels, L. T., and Tyler, F. H., 17-hydroxycorticosteroid metabolism in liver disease. J. Clin. Invest., 1954, **33**, 1524.
8. Peterson, R. E., The physiological disposition and metabolic fate of corticosterone in man, In preparation.
9. Eik-Nes, K., Sandberg, A. A., Tyler, F. H., and Samuels, L. T., Plasma levels of 17-hydroxycorticosteroids following the administration of adrenal steroids. Acta endocrinol., 1955, **18**, 244.
10. Peterson, R. E., Metabolism of some of the newer synthetic steroids, Unpublished observations.
11. Hechter, O., Solomon, M. M., and Caspi, E., Corticosteroid metabolism in liver: Studies on perfused rat livers. Endocrinology, 1953, **53**, 202.
12. Caspi, E. Y., Levy, H., and Hechter, O. M., Cortisone metabolism in liver. II. Isolation of certain cortisone metabolites. Arch. Biochem. & Biophys., 1953, **45**, 169.
13. Schneider, J. J., and Horstmann, P. M., Effects of incubating compound E and related steroids with various surviving rat tissues. J. Biol. Chem., 1952, **196**, 629.
14. Eisenstein, A. B., Steroid compounds resulting from incubation of cortisone with surviving liver slices. Proc. Soc. Exper. Biol. & Med., 1953, **83**, 27.
15. Fish, C. A., Hayano, M., and Pincus, G., Conversion of cortisone to 17-hydro-corticosterone by liver homogenates. Arch. Biochem. & Biophys., 1953, **42**, 480.
16. Amelung, D., Hübener, H. J., Roka, L., and Meyerheim, G., Conversion of cortisone to compound F. J. Clin. Endocrinol. & Metab., 1953, **13**, 1125.
17. Cope, C. L., and Hurlock, B., Some aspects of adrenal cortical metabolism. Clin. Sc., 1954, **13**, 69.
18. Burstein, S., Savard, K., and Dorfman, R. I., The *in vivo* metabolism of cortisone. Endocrinology, 1953, **52**, 448.
19. Burton, R. B., Keutmann, E. H., and Waterhouse, C., The conversion of cortisone acetate to other alpha-ketolic steroids. J. Clin. Endocrinol. & Metab., 1953, **13**, 48.
20. Ward, L. E., Polley, H. F., Slocumb, C. H., and Hench, P. S., Comparative effects of hydrocortisone, hydrocortisone acetate, and cortisone acetate in rheumatoid arthritis. J. Lab. & Clin. Med., 1952, **40**, 959.
21. Boland, E. W., Antirheumatic effects of hydrocortisone (free alcohol), hydrocortisone acetate, and cortisone (free alcohol), as compared with cortisone acetate; results from oral administration in patients with rheumatoid arthritis. Brit. M. J., 1952, **1**, 559.
22. Kupperman, H. S., Blatt, M. H. G., Vessell, M., Gagliani, J., Weisbader, H., and Vosburgh, L., The comparative effects of metacortandracin, 9-α-fluorohydrocortisone and hydrocortisone upon ACTH secretion in man. J. Clin. Endocrinol. & Metab., 1955, **15**, 911.
23. Stephenson, N. R., The relative potency of adrenal corticoids by the thymus involution method. Canad. J. Biochem. & Physiol., 1956, **34**, 253.
24. Wilson, H., Fairbanks, R., Scialabba, D., McEwen, C., and Ziff, M., Metabolites of hydrocortisone and cortisone in synovial fluid in rheumatoid arthritis. J. Clin. Endocrinol. & Metab., 1956, **16**, 86.
25. Hollander, J. L., Brown, E. M., Jr., Jessar, R. A., and Brown, C. Y., Hydrocortisone and cortisone injected into arthritic joints; comparative effects of and use of hydrocortisone as a local anti-arthritic agent. J. A. M. A., 1951, **147**, 1629.

26. Ziff, M., Scull, E., Ford, D., McEwen, C., and Bunim, J. J., Effects in rheumatoid arthritis of hydrocortisone and cortisone injected intra-articularly. Arch. Int. Med., 1952, 90, 774.
27. Dixon, A. St. J., and Bywaters, E. G. L., The effect of intra-articular injection of cortisone acetate and of hydrocortisone acetate in rheumatoid arthritis. Clin. Sc., 1953, 12, 15.
28. Peterson, R. E., Unpublished observations.
29. Ingle, D., Adrenal Cortex, Third Josiah Macy, Jr. Foundation Conference, 1951, p. 107.
30. Ingle, D. J., Nezamis, J. E., and Morley, E. H., Effect of adrenal cortical extract, cortisone and hydrocortisone upon the work of the adrenalectomized-eviscerate rat. Endocrinology, 1953, 53, 582.
31. Bondy, P. K., Ingle, D. J., and Meeks, R. C., Influence of adrenal cortical hormones upon the level of plasma amino acids in eviscerate rats. Endocrinology, 1954, 55, 354.
32. Dulin, W. E., Bowman, B. J., and Stafford, R. O., Effects of 2-methylation on glucocorticoid activity of various C-21 steroids. Proc. Soc. Exper. Biol. & Med., 1957, 94, 303.
33. Hellman, L., Bradlow, H. L., Adesman, J., Fukushima, D. K., Kulp, J. L., and Gallagher, T. F., The fate of hydrocortisone-4-C^{14} in man. J. Clin. Invest., 1954, 33, 1106.
34. Migeon, C. J., Sandberg, A. A., Decker, H. A., Smith, D. F., Paul, A. C., and Samuels, L. T., Metabolism of 4-C^{14}-cortisol in man: Body distribution and rates of conjugation. J. Clin. Endocrinol. & Metab., 1956, 16, 1137.
35. Hellman, L., Bradlow, H. L., Frazell, E. L., and Gallagher, T. F., Tracer studies of the absorption and fate of steroid hormones in man. J. Clin. Invest., 1956, 35, 1033.
36. Reddy, W. J., Jenkins, D., and Thorn, G. W., Estimation of 17-hydroxycorticoids in urine. Metabolism, 1952, 1, 511.
37. Sandberg, A. A., Nelson, D. H., Glenn, E. M., Tyler, F. H., and Samuels, L. T., 17-hydroxycorticosteroids and 17-ketosteroids in urine of human subjects: Clinical application of a method employing β-glucuronidase hydrolysis. J. Clin. Endocrinol. & Metab., 1953, 13, 1445.
38. Vestergaard, P., Investigations on the estimation of 17-hydroxycorticoids in urine using the Porter/Silber reaction. Acta endocrinol., 1953, 13, 241.
39. Fukushima, D. K., Leeds, N. S., Bradlow, H. L., Kritchevsky, T. H., Stokem, M. B., and Gallagher, T. F., The characterization of four new metabolites of adrenocortical hormones. J. Biol. Chem., 1955, 212, 449.

THYROID–ADRENOCORTICAL INTERRELATIONS: FAILURE TO DEMONSTRATE ANTAGONISM BETWEEN TRIIODO-THYRONINE AND HYDROCORTISONE IN MAN [1]

By KENNETH FREMONT-SMITH,[2] FRANK L. IBER,[3] AND IRVIN C. PLOUGH [4]

(From the Department of Metabolism, Division of Medicine, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.)

(Submitted for publication June 20, 1956; accepted May 27, 1957)

It is generally accepted that acute injury results in an outpouring of adrenocortical hormones [5] (1, 2), and that these hormones play an important role in the response of the total organism to such injury. The marked increase in urinary nitrogen excretion which characteristically occurs immediately following acute injury is usually considered to result from the catabolic action of the adrenocortical hormones on protein metabolism (3). Supporting this concept are recent studies correlating the negative nitrogen balance following surgical trauma in man with the rise in 17-hydroxysteroid excretion (4).

The participation of the thyroid gland in the response to acute injury in man is less well delineated. Certain observations have suggested that there may be an increase in circulating thyroid hormone in man following trauma (5, 6); other investigators have been unable to confirm this finding (7, 8). The role of the thyroid is particularly difficult to evaluate following injury because the increased output of adrenocortical hormones may affect some of the methods used to assay thyroid function (9–11), and may also tend to inhibit thyroid activity (9, 11–14).

Goldenberg, Lutwak, Rosenbaum, and Hayes (15) have recently proposed that the catabolic response to injury is due primarily to excess thyroid hormone rather than adrenocortical hormones. They further postulate that the adrenocortical hormones are antagonistic to the effects of excess thyroid activity on protein metabolism. According to this thesis, the increased output of adrenocortical hormones following acute injury tends to decrease the catabolism and nitrogen-wasting caused by excess thyroid activity, and thus acts to restore nitrogen balance and homeostasis. The clinical observations and experimental evidence marshalled by Goldenberg *et al.* (15, 16) in support of this hypothesis, however, have appeared inconclusive to us as well as to others (17). We decided to determine directly whether large amounts of adrenocortical and thyroid hormones are antagonistic in their metabolic effects in normal man.

l-Triiodothyronine [6] and hydrocortisone were selected as representative hormones of the thyroid and the adrenal cortex, respectively (18, 19). Each hormone was administered separately in large dosage, 1 mg. and 200 mg. daily by mouth, respectively, for three or four days to two normal human males (subjects Nos. 1 and 2); subsequently, both hormones were administered simultaneously at the same dosage as before. Evidence for mutual antagonism between the two hormones was sought in the nitrogen, potassium and sodium balances; urinary steroid excretion was also followed. A further study was carried out on a patient who had undergone adrenalectomy for Cushing's syndrome five months before and who repeatedly failed to respond to adrenocorticotropic hormone postoperatively (subject No. 3).[7]

[1] Presented in part before the Eastern Section of the American Federation for Clinical Research, Philadelphia, Pennsylvania, December 2, 1955.

[2] Formerly Captain, MC, USAR. Present Address: Mary Imogene Bassett Hospital, Cooperstown, New York.

[3] Formerly Captain, MC, USAR. Present Address: Johns Hopkins Hospital, Baltimore, Maryland.

[4] Lt. Col., MC, USA. Present Address: Medical Nutrition Laboratory, Fitzsimons Army Hospital, Denver, Colorado.

[5] Throughout this paper the term "adrenocortical hormones" is used to refer exclusively to the glucocorticoids.

[6] Kindly provided by Dr. Frederick K. Heath, Merck & Co., Inc., Rahway, New Jersey.

[7] This patient will be reported in detail elsewhere. A solitary left adrenal adenoma was removed at operation together with the atrophic left adrenal gland; no adrenal gland could be found on the right. Postoperatively he received maximal stimulation with ACTH on five separate occasions (four and three months before, and one, two and four months after the studies reported in this

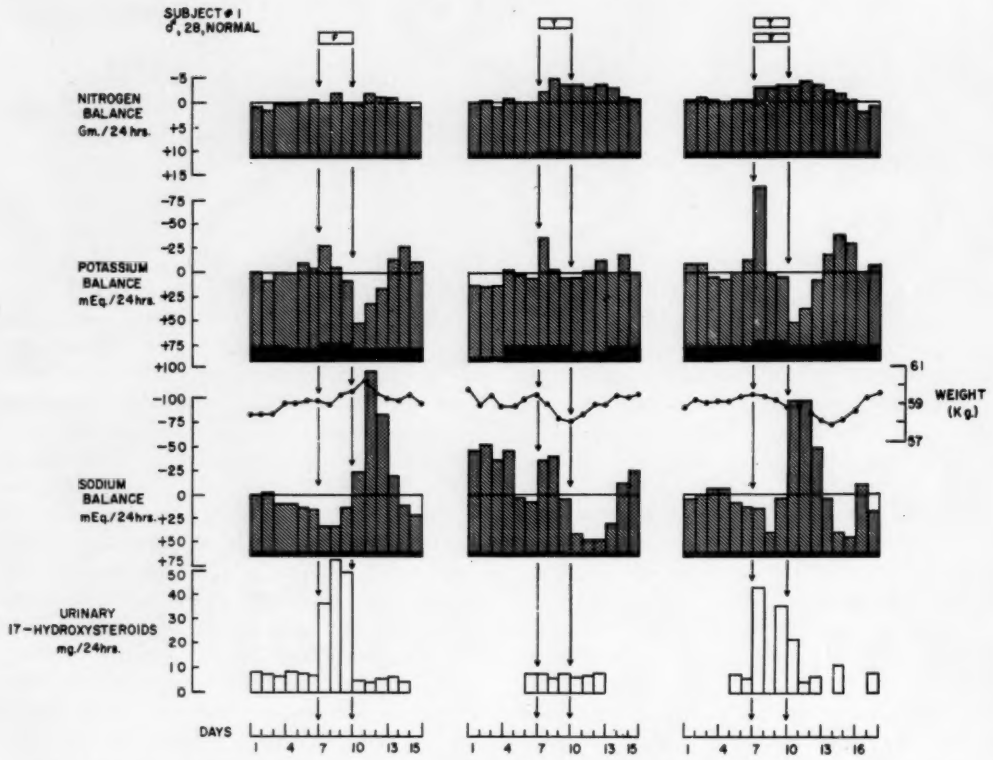


FIG. 1. BALANCE STUDIES IN SUBJECT NO. 1 (NORMAL), CHARTERED AFTER REIFENSTEIN *ET AL.* (21)

Equilibration periods not shown; no equilibration period obtained before the l-triiodothyronine study. F = Hydrocortisone Acetate (Compound F), 50 mg. orally every 6 hours; T = l-Triiodothyronine, 0.5 mg. orally every 12 hours.

METHODS

Prolonged metabolic balance studies were conducted on the Metabolic Ward of Walter Reed Army Hospital. Balance procedures employed in this department have been described previously (20). Liquid diets, identical from day to day, were used throughout except for a two-day period during the study of subject No. 3 (see Results). Diets for the two normal subjects were adequate in calories (50 Cal. per Kg.) and protein (1.3 and 1.5 Gm. per Kg.); subject No. 3 was maintained on a diet adequate in protein (0.9 Gm. per Kg.) but inadequate in calories (10 Cal. per Kg.) because of residual obesity. Urine collection periods were 24 hours in duration with the exception of several 48-hour periods in the study of subject No. 3; completeness of urinary collections was checked by daily urinary creatinine determinations. Stool collection periods were 72 or 96 hours in duration. The balance data are charted according to Reifenstein, Albright, and Wells (21); the zero line is constructed as the average of the control balance periods. Equilibration periods of two to seven days' duration (not shown in the figures) were obtained before each study of subjects Nos. 1 and 2 with one exception.[8] Subject No. 3 had been on the identical reducing diet for over a month at the time of the start of his study.

Standard methods were employed for the chemical analyses (22). The urinary 17-ketosteroid determinations were made by a modification of the Zimmerman reaction (23) and the 17-hydroxysteroids by a modification (24) of the Porter-Silber method (25).

RESULTS

The results of the balance studies in the two normal subjects are shown in Figures 1 and 2. In

paper) for periods ranging from three to ten days. At no time did the urinary steroid excretion reveal any response whatsoever. We have been forced to conclude that the patient has no remaining adrenocortical tissue.

[8] There was no equilibration period prior to the triiodothyronine study in subject No. 1.

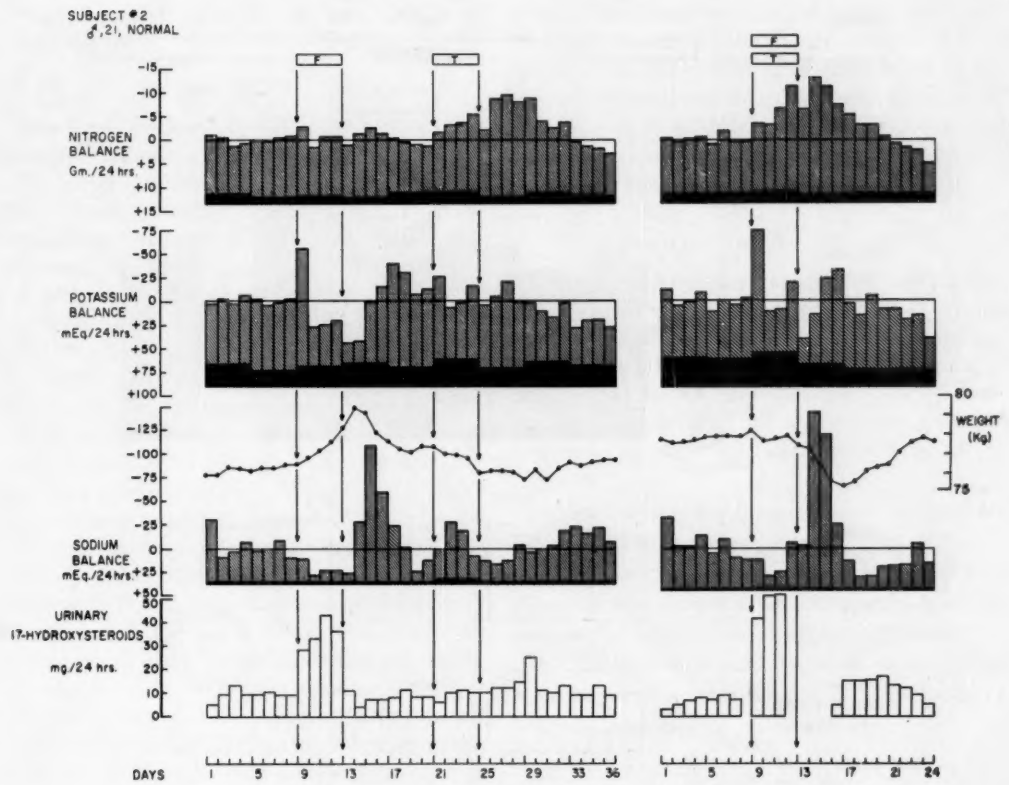


FIG. 2. BALANCE STUDIES IN SUBJECT NO. 2 (NORMAL)

Equilibration periods not shown. F = Hydrocortisone Acetate (Compound F), 50 mg. orally every 6 hours; T = l-Triiodothyronine, 0.5 mg. orally every 12 hours.

each study, hydrocortisone had little effect on the nitrogen balance, whereas triiodothyronine resulted in a prolonged period of nitrogen loss in the urine. When both hormones were administered together, the resulting negative nitrogen balance was at least as great as that produced by the triiodothyronine alone (Table I).

The balances of potassium and sodium showed the expected changes during hydrocortisone administration, but were not consistently affected by triiodothyronine. When both hormones were given simultaneously, the results were essentially the same as those observed with hydrocortisone alone.

The excretion of urinary 17-hydroxysteroids increased as expected with each administration of hydrocortisone in both normal subjects. In subject No. 1 there was no apparent change with triiodothyronine administration, although observations were not extended throughout the recovery period (Figure 1). Subject No. 2, however, showed a small but definite rise in 17-hydroxysteroid excretion during the five days immediately following triiodothyronine administration, approx-

TABLE I

Cumulative negative nitrogen balance (Gm.) resulting from hormone administration

	Hydrocortisone*	1-Triiodothyronine†	Hydrocortisone* and 1-Triiodothyronine†
Subject No. 1	10.0	23.9	23.0
Subject No. 2	5.4	59.0	73.4

* 200 mg. daily.
† 1.0 mg. daily.

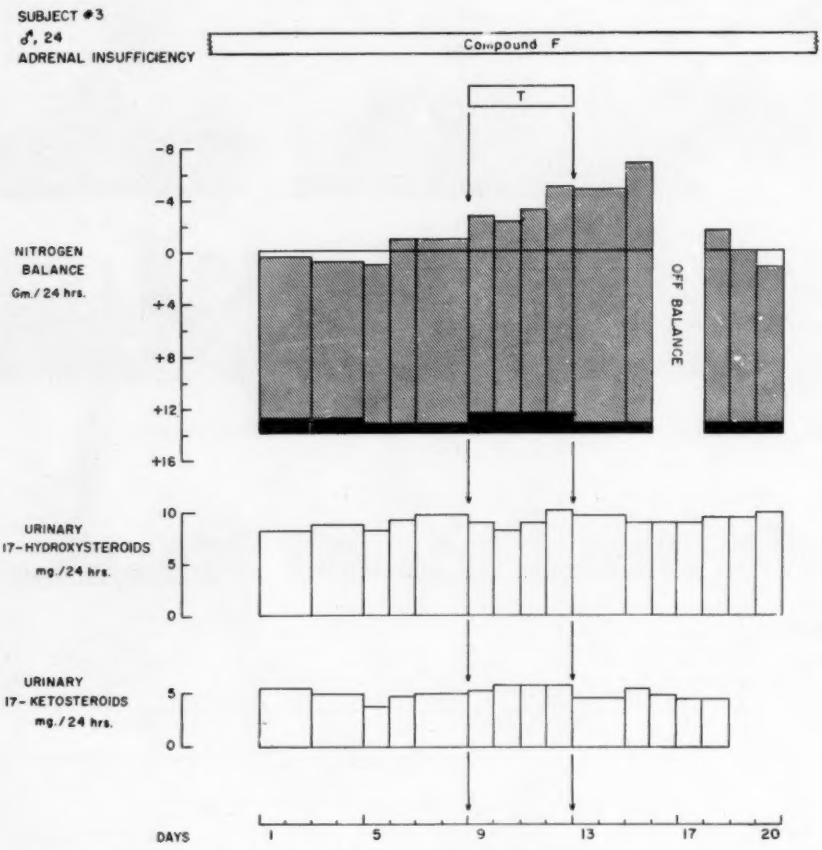


FIG. 3. BALANCE STUDY IN SUBJECT NO. 3 (TOTAL ADRENOCORTICAL INSUFFICIENCY)

Equilibration period not shown. Compound F = Hydrocortisone Acetate, 10 mg. orally every 6 hours; T = l-Triiodothyronine, 0.5 mg. orally every 12 hours. See foot-note number 10 for explanation of period off balance.

imately coinciding with the period of maximal nitrogen excretion (Figure 2). This slight increase was also seen in this subject during the recovery period following the simultaneous administration of both hormones. It appeared possible that triiodothyronine administration had resulted in an increased output of adrenocortical hormones in this subject; had this been the case, the negative nitrogen balance following triiodothyronine might have been due in part to adrenocortical stimulation. An alteration in peripheral utilization, degradation or renal excretion of adrenal hormones presented alternative possibilities.

Subject No. 3 was studied with these questions in mind. This patient was maintained throughout the study (and for one month previously) on hydrocortisone [9] alone, his only source, exogenous or endogenous, of glucocorticoids. When triiodothyronine was administered in the same dosage as to the normal subjects, a period of negative nitrogen balance resulted entirely similar to that seen in the normal subjects (Figure 3) [10] and was

[9] 10 mg. every six hours by mouth.

[10] At the peak of the period of negative nitrogen balance (72 hours after stopping triiodothyronine), there was such a marked increase in this patient's sense of lassitude and fatigue that the balance study had to be discontinued for 48 hours for morale purposes. Complete urinary collections for steroid determinations were con-

not accompanied by any significant change in steroid excretion.

DISCUSSION

The thesis proposed by Goldenberg *et al.*, that adrenocortical hormones may have anabolic activity in the presence of increased thyroid hormone, deserves careful scrutiny. Both adrenocortical hormones and thyroid hormone are usually considered to have catabolic effects on protein metabolism when present in excess (*27*). If the concept under consideration were correct, it would prove that the effects of these two hormones at a cellular or enzyme level, hitherto thought to be independent, are instead closely interrelated.

Goldenberg and co-workers (15) have submitted metabolic balance data following operative procedures and during the administration of desiccated thyroid, propylthiouracil, ACTH, and adrenocortical extract in various combinations (16) in support of their theory of thyroid-adrenocortical antagonism in man. Certain changes in urinary excretion of nitrogen and other metabolites have been interpreted as representing major and significant alterations in metabolism in accord with their concept. Scrutiny of the balance charts suggests to the present authors that these changes are often consistent with minor fluctuations in baseline of the type which plague all balance studies. In our opinion, their data as presented do not strongly support their thesis.

The metabolic studies in the two normal subjects reported in this paper failed to show antagonistic action between large doses of adrenocortical hormone (hydrocortisone) and of thyroid hormone (triiodothyronine) by the criteria of nitrogen, potassium and sodium balance. Simultaneous administration of both hormones produced responses as great as those produced by either alone. The study done on the patient with complete adrenocortical insufficiency (subject No. 3) revealed that the protein catabolism caused by excess triiodothyronine does not depend upon an increased output of adrenocortical hormones, and that the urinary excretion of exogenous hydrocortisone is not grossly altered by triiodothyronine.

SUMMARY AND CONCLUSIONS

1. Studies designed to demonstrate antagonistic metabolic effects between large doses of hydrocortisone and l-triiodothyronine in two normal human subjects have been negative with regard to nitrogen, potassium and sodium balance. Simultaneous administration of both hormones produced responses as great as those produced by either alone.

2. Studies on a patient without endogenous adrenocortical hormones revealed that:

a. An increased output of adrenocortical hormones is not necessary for the protein catabolic response to large doses of l-triiodothyronine.

b. The rate of urinary excretion of exogenous hydrocortisone is uninfluenced by large doses of l-triiodothyronine.

ACKNOWLEDGMENT

We are indebted to J. Long, Captain, ANC, and her nursing staff; to M. E. Shipman, 1st Lt., F. M. Berger, Captain, AMSC, and their dietary staff; and to Mr. William Alexander for his technical assistance.

ADDENDUM

Since this paper was submitted for publication, subject No. 3 received a sixth course of ACTH stimulation, ten months after completion of the studies reported in this paper. As with several of the previous courses of ACTH, he was maintained throughout the period of maximal stimulation on 2 mg. of 9-α-fluorohydrocortisone daily. For the first time there was a slight but definite response in the urinary excretion of 17-hydroxysteroids, from an average control value of 0.9 mg. per 24 hours to 3.7 mg. per 24 hours after five days of intravenous ACTH administration. Thus, contrary to our prior belief, this patient does have remaining viable adrenocortical tissue; however, it was demonstrably impossible to elicit a response from this small adrenal remnant at the time of the studies herein reported. The premise that he was unable to elaborate endogenous glucocorticoids at the time of the studies here reported would still appear valid.

tinued, however. Physical examination provided no clue as to the cause of the patient's symptoms. Serum electrolytes were within normal limits. Recovery was complete within 12 hours on a free, solid diet without caloric restriction; no medications were administered. It is interesting that both normal subjects experienced similar symptoms, although not nearly so severe, at the height of the negative nitrogen balance. Conceivably hypomagnesemia may have been responsible for these symptoms (*26*); unfortunately serum magnesium determinations were not made.

REFERENCES

1. Sayers, G., Adrenal cortex and homeostasis. Physiol. Rev., 1950, **30**, 241.
2. Sandberg, A. A., Eik-Nes, K., Samuels, L. T., and Tyler, F. H., The effects of surgery on the blood levels and metabolism of 17-hydroxycorticosteroids in man. J. Clin. Invest., 1954, **33**, 1509.
3. Moore, F. D., and Ball, M. R., The Metabolic Response to Surgery. Springfield, Charles C Thomas, 1952.
4. Moore, F. D., Steenburg, R. W., Ball, M. R., Wilson, G. M., and Myrden, J. A., Studies in surgical endocrinology. I. The urinary excretion of 17-hydroxycorticoids, and associated metabolic changes, in cases of soft tissue trauma of varying severity and in bone trauma. Ann. Surg., 1955, **141**, 145.
5. Shipley, R. A., and MacIntyre, F. H., Effect of stress, TSH and ACTH on the level of hormonal I^{131} of serum. J. Clin. Endocrinol. & Metab., 1954, **14**, 309.
6. Goldenberg, I. S., Lutwak, L., Rosenbaum, P. J., and Hayes, M. A., Thyroid activity during operation. Surg., Gynec. & Obst., 1956, **102**, 129.
7. Perry, W. F., and Gemmell, J. P., The effect of surgical operations on the excretion of iodine, corticosteroids, and uric acid. Canad. J. Research (Section E), 1949, **27**, 320.
8. Engstrom, W. W., and Markardt, B., The effects of serious illness and surgical stress on the circulating thyroid hormone. J. Clin. Endocrinol. & Metab., 1955, **15**, 953.
9. Berson, S. A., and Yalow, R. S., The effect of cortisone on the iodine accumulating function of the thyroid gland in euthyroid subjects. J. Clin. Endocrinol. & Metab., 1952, **12**, 407.
10. Ingbar, S. H., The effect of cortisone on the thyroidal and renal metabolism of iodine. Endocrinology, 1953, **53**, 171.
11. Paris, J., Ford, E., Lorenz, N., Keating, F. R., Jr., and Albert, A., Effect of hormones on renal clearance of radioiodine in the rat. Am. J. Physiol., 1955, **183**, 163.
12. Hill, S. R., Jr., Reiss, R. S., Forsham, P. H., and Thorn, G. W., The effect of adrenocorticotropin and cortisone on thyroid function: thyroid-adrenocortical interrelationships. J. Clin. Endocrinol. & Metab., 1950, **10**, 1375.
13. Wolfson, W. Q., Beierwalters, W. H., Robinson, W. D., Duff, I. F., Jones, J. R., Knorpp, C. T., and Eya, M., Corticogenic hypothyroidism: its regular occurrence and clinical significance during prolonged therapeutic administration of ACTH or cortisone (abstract). J. Lab. & Clin. Med., 1950, **36**, 1005.
14. Fredrickson, D. S., Forsham, P. H., and Thorn, G. W., The effect of massive cortisone therapy on measurements of thyroid function. J. Clin. Endocrinol. & Metab., 1952, **12**, 541.
15. Goldenberg, I. S., Lutwak, L., Rosenbaum, P. J., and Hayes, M. A., Thyroid-adrenocortical interrelations following operation. Surg., Gynec. & Obst., 1954, **98**, 513.
16. Goldenberg, I. S., Lutwak, L., Rosenbaum, P. J., and Hayes, M. A., Thyroid-adrenocortical metabolic interrelations. J. Clin. Endocrinol. & Metab., 1955, **15**, 227.
17. Thyroid-adrenocortical interrelations. Nutrition Rev., 1954, **12**, 265.
18. Gross, J., and Pitt-Rivers, R., Physiological activity of 3:5:3'-L-triiodothyronine. Lancet, 1952, **2**, 593.
19. Romanoff, E. B., Hudson, P., and Pincus, G., Isolation of hydrocortisone and corticosterone from human adrenal vein blood. J. Clin. Endocrinol. & Metab., 1953, **13**, 1546.
20. Forsyth, B. T., Shipman, M. E., and Plough, I. C., The variability of fecal nitrogen excretion with liquid and solid diets. J. Lab. & Clin. Med., 1954, **43**, 440.
21. Reifenstein, E. C., Jr., Albright, F., and Wells, S. L., The accumulation, interpretation, and presentation of data pertaining to metabolic balances, notably those of calcium, phosphorus, and nitrogen. J. Clin. Endocrinol. & Metab., 1945, **5**, 367.
22. Knowlton, M., Horner, W. H., Seligson, D., and Iber, F. L., Analytical Procedures. Washington, U. S. Government Printing Office, 1955.
23. M. R. C. Committee on Clinical Endocrinology: Proposed standard method of 17-ketosteroid determination. Lancet, 1951, **1**, 585.
24. Petersen, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B., and Bunim, J. J., The physiological disposition and metabolic fate of hydrocortisone in man. J. Clin. Invest., 1955, **34**, 1779.
25. Porter, C. C., and Silber, R. H., A quantitative color reaction for cortisone and related 17,21-dihydroxy-20-ketosteroids. J. Biol. Chem., 1950, **185**, 201.
26. Tapley, D. F., Magnesium balance in myxedematous patients treated with triiodothyronine: preliminary note. Bull. Johns Hopkins Hosp., 1955, **96**, 274.
27. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry. Interpretations, Volume I, 2nd ed. Baltimore, Williams and Wilkins Co., 1946, page 668.

THE INFLUENCE OF AUTONOMIC VASOREGULATORY REFLEXES ON THE RATE OF SODIUM AND WATER EXCRETION IN MAN

By HENRY N. WAGNER, JR.

(From the National Heart Institute, National Institutes of Health, Bethesda, Md.)

(Submitted for publication March 27, 1957; accepted May 28, 1957)

In man, the function of the autonomic nervous system has been studied in two principal ways: 1) by determining the physiologic effects of paravertebral sympathectomy; and 2) by simulating or inhibiting the action of the autonomic nerves pharmacologically. In this study, a third approach has been used in an attempt to determine the role of the autonomic nervous system in physiologic processes. Studies were made of patients with marked autonomic hypofunction, manifested chiefly by orthostatic hypotension, diminished sweating and impotence. The patients studied had no other associated condition, such as generalized debility, Addison's disease, diabetes mellitus, syphilis, amyloidosis or somatic neurological disease, to which the autonomic dysfunction could be attributed. The disease process was very stable, changes occurring only over a period of several months or even years. In these patients it was found that the defect involved the parasympathetic as well as the sympathetic nervous systems (1). The cardiovascular, gastrointestinal, genitourinary and thermoregulatory systems were affected.

The purpose of this report is to present the results of studies of the hemodynamic and renal effects of acute changes in blood and extracellular fluid volume in these patients.

DESCRIPTION OF PATIENTS

Studies were performed in five patients and seven normal persons. A brief clinical description of three of the patients has been given in a previous report (1). Two additional patients have been studied. Patient L. S. (NIH 01-27-85) was a 21-year-old college student. Twenty-seven months prior to admission, he developed generalized weakness, anorexia and diarrhea. On standing, he complained of dizziness and blurring of vision. He also noted a marked watery nasal and post-nasal discharge associated with nasal stuffiness. There was occasional excessive lacrimation and salivation with pain in the region of the parotid and submaxillary salivary glands, aggravated by eating. For several months he was bedridden with postural hypotension so severe that he had difficulty sitting up in bed. Upon the arrival of summer, five months after the onset of his illness, he noted that he no longer sweated, in marked contrast to the increased sweat gland activity that he noted at the beginning of his illness. Impotence and impaired ejaculations were also noted at about the same time. In the first month of illness, he had difficulty in micturition and defecation. Early in his illness, he was found to have an elevated spinal fluid protein (99 mg. per cent) which gradually fell to normal in two months. There were no other spinal fluid abnormalities. Of possible significance is the fact that a slightly papular, erythematous, pruritic rash appeared over his trunk during the first month of his illness.

After the first year of illness, the diarrhea, anorexia and postural symptoms improved somewhat. At the time of admission to the National Institutes of Health, 27 months after the onset of symptoms, he complained of chronic diarrhea and extreme generalized weakness, in addition to blurring of vision and syncope on standing. Positive physical findings were emaciation, slight pectus excavatum, and orthostatic hypotension. General and neurological examination were otherwise normal. Urinalysis and hemogram were within normal limits. Spinal fluid examinations were normal. Urinary 17-ketosteroid and plasma hydrocortisone levels were normal.

Patient M. H. (NIH No. 01-36-95) was a 58-year-old retired mail carrier. At age 46 he noticed sudden loss of sweating over the entire body with the exception of the axillae. At the same time he noted heat intolerance. Both have persisted essentially unchanged for the past 12 years. Eleven years ago he developed generalized weakness, aggravated by both erect posture and warm environmental temperatures. He had syncopal attacks associated with hyperventilation and clonic

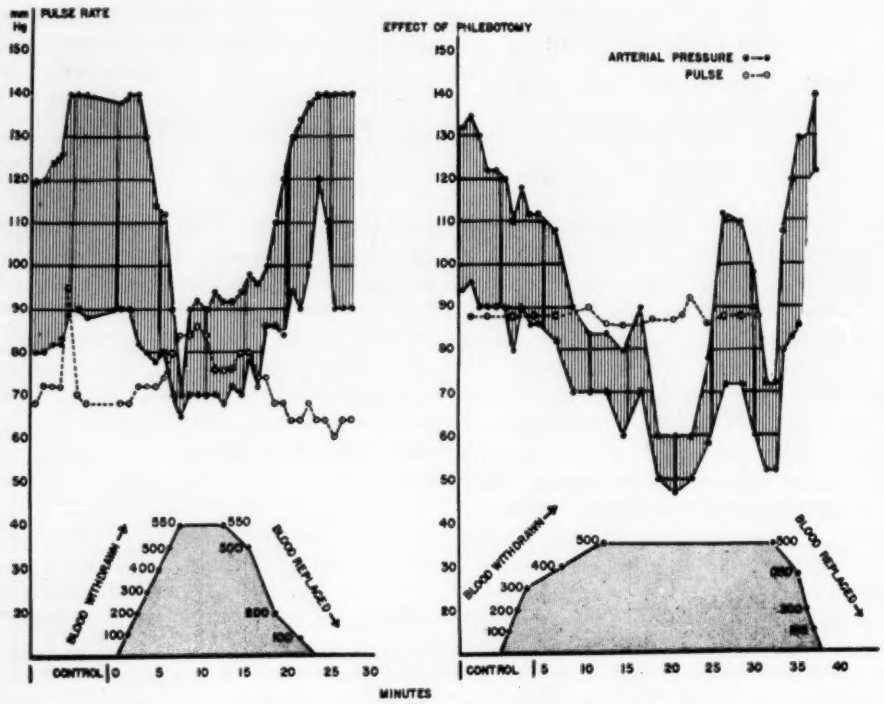


FIG. 1. THE EFFECT OF RAPID REMOVAL AND REPLACEMENT OF WHOLE BLOOD ON THE ARTERIAL PRESSURE OF TWO PATIENTS WITH POSTURAL HYPOTENSION DURING RECUMBENCY

movements of the extremities without incontinence. For two to three years prior to admission, the weakness had become more pronounced. Nine years prior to admission he noted impaired ejaculation progressing to complete impotence during the subsequent four years. Two years before admission he developed marked constipation to the extent that he has periods as long as five to seven days without bowel movements. The patient has not had diarrhea at any time during his illness. For the past two years, he has had urinary urgency and hesitancy. In July, 1947, the diagnosis of postural hypotension was made. At that time, his arterial pressure in the recumbent position was 130/82, falling to 84/62 on standing.

Positive physical findings were an arterial pressure of 180/110, falling to 72/54 on passive standing. He appeared older than 58 years. The skin was extremely dry and flaky. Nasal septum was perforated. No other abnormalities of the general or neurological physical examination were present, except as related to autonomic nervous system function. Urinalysis and hemogram were within normal limits. Plasma hydrocortisone level and response to ACTH were normal.

In all patients, graded passive tilting resulted in progressively lowered arterial pressure. In patient L.S. a rise in pulse rate occurred simultaneously. In patient M.H. no increase in pulse rate occurred with the fall in arterial pressure.

Involvement of the sympathetic nervous system of these patients was indicated by the diminished sweating, postural hypotension and absence of hypertensive "overshoot" following Valsalva maneuver. Abnormalities of the parasympathetic nervous system were suggested by: 1) the failure of atropine to alter the pulse rate; 2) the absence of bradycardia during the administration of norepinephrine; 3) the lack of effect on the pulse rate and arterial pressure of carotid sinus massage;

4) the inability to have erections; and 5) the failure of hypoglycemia to produce an increase in gastric acidity.[1]

METHODS AND MATERIALS

Brachial arterial pressures were measured by means of a sphygmomanometer. Mean arterial pressure was calculated as one-third of the pulse pressure plus diastolic pressure. In the studies in which whole blood was infused, mean pressure was recorded by means of a mercury manometer attached to an indwelling needle in the femoral artery. Glomerular filtration rate was measured by means of the clearance of inulin (2). Urine was collected by means of an indwelling rubber urethral catheter. At the beginning and end of each collection period, the bladder was emptied with 40 ml. of air, unless the urine flow was greater than 10 ml. per minute. The duration of the urine collection periods varied from 10 to 60 minutes. Osmolal concentration of urine and plasma was determined by the method of freezing-point depression. Sodium and potassium were measured by internal standard flame photometry. The sodium-retaining steroid used was 2-methyl, 9-alpha fluorohydrocortisone (Upjohn) (3). Purified arginine vasopressin and Pitressin® (Parke-Davis) were also studied.

All studies were performed with the patients recumbent throughout. They were begun at approximately 9:00 A.M. Both patients and normal subjects were on an *ad libitum* diet prior to the studies. All subjects and patients were deprived of food and water for from 13 to 16 hours prior to the studies of renal function. The ages of the control subjects ranged from 18 to 72 years; the patients, from 21 to 66.

RESULTS

I. Effect on arterial pressure of changes in plasma and extracellular fluid (ECF) volume

In previous studies of orthostatic hypotension, the inability of the patients to adjust their arterial pressure to postural changes has been emphasized. However, it can be demonstrated that the fall in arterial pressure that occurs in these patients when they assume an upright posture is merely a specific manifestation of a general inability to keep arterial pressure constant in the presence of variations in blood volume.

Figure 1 illustrates the effect of rapidly removing 500 milliliters of blood from each of two patients. The patients were recumbent in these studies and in those to be described subsequently. As the blood was withdrawn, a marked fall in arterial pressure occurred. When the blood was reinfused, the pressure returned to normal levels.

TABLE I

Pressor response to acute hypervolemia

	Control mean pressure*	Post-infusion mean pressure†
Whole blood‡ (500 ml.)	67	116
Normal saline§ (3,000 ml.)	85	108
Albumin‡ (50 Gm.) (200 ml.)	64	101

* Average of pressures recorded every three minutes for one hour prior to the beginning of the infusion.
† Mean of the pressures obtained every three minuest for one hour.
‡ Infused over a period of about 20 minutes.
§ Infused over a period of 60 minutes.

In addition, in these patients arterial pressure increased markedly when blood volume or ECF volume was suddenly expanded. In Table I is shown the effect of the rapid infusion of whole blood,[2] normal saline and albumin on the arterial pressure of one patient during recumbency. A marked increase in arterial pressure occurred in each case. In all patients, except one, similar results were obtained. The exception was the patient M. H., who was hypertensive in the recumbent position whenever studied. In this patient, the infusion of blood, saline or albumin did not significantly increase arterial pressure. In all patients, if the arterial pressure was elevated to hypertensive levels by means of vasopressin or adrenal steroid administration prior to expansion of plasma or ECF volume, no further increase in arterial pressure occurred. For example, in Table II are shown the results of acute expansion of the ECF volume with saline during the course of chronic 2-methyl, 9-alpha fluorohydrocortisone administration. As a result of the methyl fluorohydrocortisone, the arterial pressure was elevated during the control periods. When saline was rapidly infused, no further significant increase in arterial pressure occurred. Similar results were obtained when the control arterial pressure was elevated with vasopressin prior to the saline infusion.

In the normal subjects, the infusion of 3,000 ml.

[1] Not all parasympathetic defects listed were present in all five patients. All sympathetic defects were present.

[2] The whole blood administered was removed from the patient in small amounts in the week prior to its readministration.

TABLE II

*Pressure response to acute hypervolemia during methyl fluorohydrocortisone administration**

	Patient	Control mean pressure	Post-infusion mean pressure
Normal saline (3,000 ml.)	B. B.	113	120
	L. S.	118	122
	M. H.	121	126
	L. S.	116	118
Albumin (50 Gm.)	B. B.	113	118
	H. C.	131	131

* Pressures were recorded as described in Table I.

of saline did not significantly increase arterial pressure. The effect of whole blood was not studied in normal subjects.

II. Renal effects of the rapid administration of isotonic saline

In addition to the abnormal hemodynamic response of these patients to expansion of extracellular fluid volume, there was a striking difference between these patients and normal persons in their ability to excrete both sodium and water in response to the administration of isotonic sodium chloride solutions (154 mEq. per L.). In seven normal persons and in four patients, food and water were withheld for 13 to 16 hours. Isotonic sodium chloride solution was then administered at a rate of 50 ml. per minute for one hour. Table III summarizes the results of these studies. During the infusion of isotonic saline to the patients in the recumbent position, average urine flow was 23 ml. per minute, ranging from 18 to 31 ml. per minute; sodium excretion during the hour's infusion was 80.3 mEq. per hour, ranging from 45 to 128 mEq. per hour; and the average maximum rate of sodium excretion during the infusion was 2,500 microequivalents per minute, ranging from 1,600 to 4,000. In contrast, in the normal persons studied under similar conditions, corresponding values were 6.3 ml. per minute (range, 1.4 to 12.7); 14.9 mEq. per hour (0.8 to 19.9); and 702 microequivalents per minute (220 to 1,450). The ranges of these measurements did not overlap in the two groups of persons. Figure 2 (A, B) shows the results of these experiments graphically. In part A, it may be seen that in the normal persons, a moderate increase in sodium excretion occurred in association with the infusion. In contrast, in part B it may be seen that in the four patients studied a marked and rapid increase in sodium excretion occurred, beginning during the period of the infusion.

Figure 3 (A, B) shows the effect of the infusion of isotonic saline on the excretion of solute-free water in the two groups. Solute-free water (C_{H_2O}) is defined as the amount of water excreted per minute in excess of that required to produce an isosmotic urine. It is calculated by subtracting osmolal clearance (C_{OSM}) from urine flow (V). It is positive in the case of urines that are hypotonic to plasma and negative in the case of hypertonic urines.

In addition to the enhanced sodium excretion, in the patients there was a great increase in the excretion of solute-free water as isotonic saline was infused. In all patients the urine became hypotonic. In contrast, in Figure 3, part A, it may be seen that in normal persons the urine became hypotonic in only four persons and in none of the control persons was the increase in solute-free water as great as that observed in the patients.

TABLE III

Renal response to isotonic saline administration (3,000 ml.) in normal persons and patients with idiopathic postural hypotension

	Patients (four)		Control subjects (seven)	
	Mean	Range	Mean	Range
Urine flow (ml./min.)	23	18–31	6	1–13
Sodium excretion during the hour's infusion (milliequivalents/hour)	80	45–128	15	1–20
Average maximal sodium excretion (microequivalents/minute)	2,500	1,600–4,000	702	220–1,450

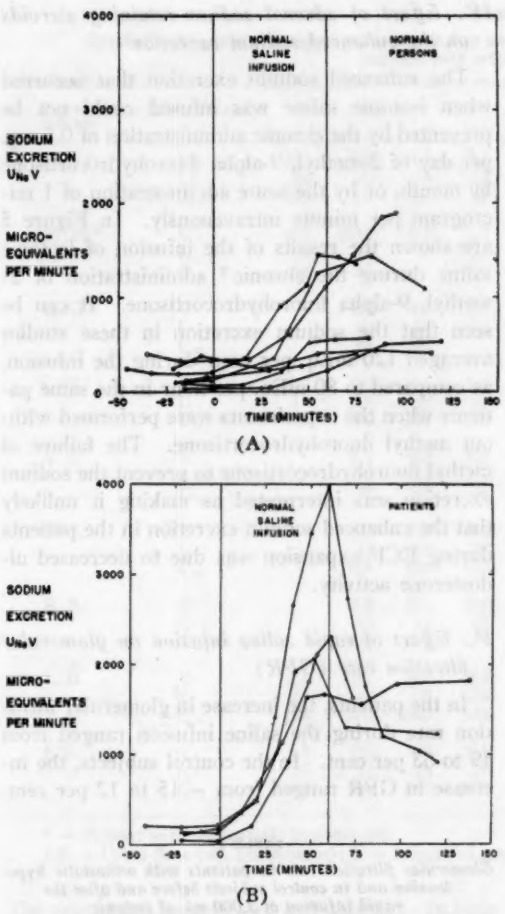


FIG. 2. THE EFFECT ON SODIUM EXCRETION OF THE INFUSION OF THREE LITERS OF ISOTONIC SODIUM CHLORIDE FOLLOWING 13 TO 16 HOURS FOOD AND WATER DEPRIVATION

The persons were recumbent throughout the study. A refers to 7 normal subjects; B refers to identical studies in 4 persons with postural hypotension.

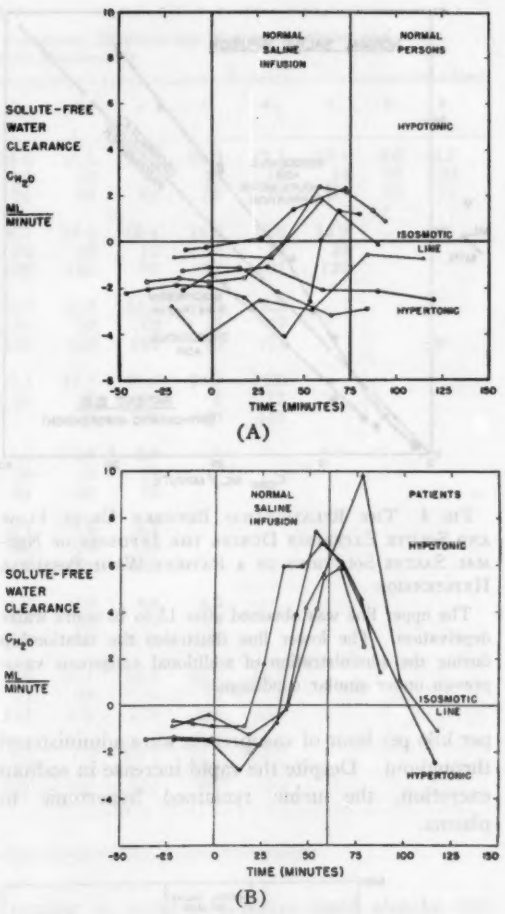


FIG. 3. THE EFFECT ON SOLUTE-FREE WATER EXCRETION OF THE INFUSION OF THREE LITERS OF ISOTONIC SODIUM CHLORIDE SOLUTIONS FOLLOWING 13 AND 16 HOURS WATER DEPRIVATION

In figure A, the infusion ended at 60 minutes, not 75 minutes. A, normal persons; B, patients with orthostatic hypotension.

III. Effect of vasopressin on the excretion of solute-free water

The transition of the urine from hypertonic to hypotonic in association with the marked increase in sodium excretion was thought to be due to insufficient antidiuretic hormone (ADH) in the presence of an acutely rising sodium excretion (4). Accordingly, experiments were performed in which exogenous vasopressin was administered to supplement the endogenous ADH resulting from the 13 to 16 hour water deprivation. An example of such a study is seen in Figure 4. In this patient, food and water were withheld for 13 hours. Dehydration and the associated endogenous ADH release resulted in the formation of hypertonic urines. As solute excretion then increased acutely during the infusion of isotonic saline, the urine became hypotonic and solute-free water clearance became positive. On another day (lower line on Figure 4), the experiment was repeated, but 5 milliunits

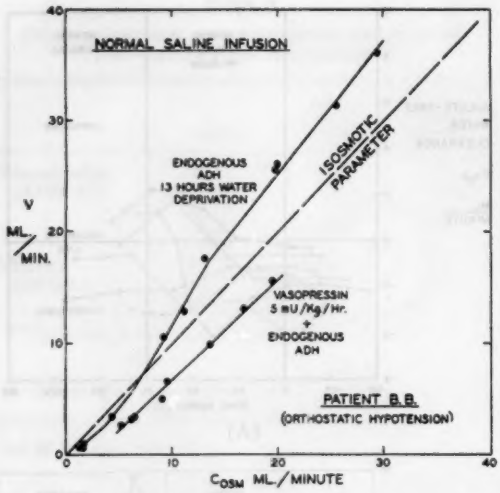


FIG. 4. THE RELATIONSHIP BETWEEN URINE FLOW AND SOLUTE EXCRETION DURING THE INFUSION OF NORMAL SALINE SOLUTIONS IN A PATIENT WITH POSTURAL HYPOTENSION

The upper line was obtained after 13 to 16 hours water deprivation. The lower line illustrates the relationship during the administration of additional exogenous vasopressin under similar conditions.

per kilo per hour of vasopressin were administered throughout. Despite the rapid increase in sodium excretion, the urine remained hypertonic to plasma.

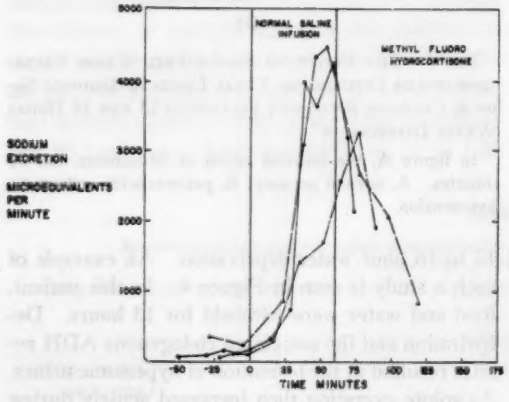


FIG. 5. THE EFFECT ON SODIUM EXCRETION OF THE INFUSION OF 3,000 ML. OF ISOTONIC SODIUM CHLORIDE SOLUTIONS DURING THE CHRONIC ADMINISTRATION OF 2-METHYL, 9-ALPHA FLUOROHYDROCORTISONE TO PATIENT WITH ORTHOSTATIC HYPOTENSION

IV. Effect of adrenal sodium-retaining steroids on the enhanced sodium excretion

The enhanced sodium excretion that occurred when isotonic saline was infused could not be prevented by the chronic administration of 0.5 mg. per day of 2-methyl, 9-alpha fluorohydrocortisone by mouth, or by the acute administration of 1 microgram per minute intravenously. In Figure 5 are shown the results of the infusion of isotonic saline during the chronic [3] administration of 2-methyl, 9-alpha fluorohydrocortisone. It can be seen that the sodium excretion in these studies averaged 120 mEq. per hour during the infusion, as compared to 80 mEq. per hour in the same patients when the experiments were performed without methyl fluorohydrocortisone. The failure of methyl fluorohydrocortisone to prevent the sodium excretion was interpreted as making it unlikely that the enhanced sodium excretion in the patients during ECF expansion was due to decreased aldosterone activity.

V. Effect of rapid saline infusion on glomerular filtration rate (GFR)

In the patients, the increase in glomerular filtration rate during the saline infusion ranged from 19 to 63 per cent. In the control subjects, the increase in GFR ranged from − 15 to 12 per cent.

TABLE IV

Glomerular filtration rate in patients with orthostatic hypotension and in control subjects before and after the rapid infusion of 3,000 ml. of isotonic sodium chloride *

	Mean of control periods	Range	Mean of periods during infusion	Range	Change %
Patient					
B. B.	56	56–57	91	79–98	63
M. H.	84	83–85	100	90–109	19
H. C.	89	87–91	106	96–110	19
L. S.	140	134–143	176	145–196	26
Subject					
V. S.	94	92–97	80	76–83	−15
G. S.	124	120–128	135	125–140	8
R. S.	131	129–132	147	132–168	12
L. B.	109	103–121	120	103–134	10
I. S.	147	140–155	160	151–170	9

* Inulin clearance determinations were performed in only five of the seven control subjects.

[3] At least three weeks of continuous administration prior to the acute study.

TABLE V

Effect of sodium chloride infusions on urine flow and glomerular filtration rate in normal persons and in patients with orthostatic hypotension

Period / Subject		−3	−2	−1	1	2	3	4	5	6	7	8	9
B. B.*	V†	0.7	0.9	0.7	3.4	25.6	31.4	36.2	26.0	17.5	10.5	6.0	5.2
	L†	10	10	10	20	10	10	10	10	10	10	30	10
	C_{in}†	56	57	56	79	96	90	98	92	74	65	72	71
M. H.*	V	0.7	0.95	0.7	2.0	8.7	17.4	18.4	16.6	15.8	13.9		
	L	10	10	9	21	20	10	10	20	20	20		
	C_{in}	83	85		101	109	104	90	97	102	120		
H. C.*	V		1.0	0.9	2.3	9.7	21.3	23.7	16.0	8.2			
	L		20	20	13	20	10	10	15	49			
	C_{in}		91	87		106	110	110	96	107			
L. S.*	V	2.2	2.0	2.1	3.4	9.5	17.7	21.2	22.9	15.0			
	L	19	19	20	20	16	15	14	8	12			
	C_{in}	142	134	143	170		176	196	169	145			
V. S.	V		1.1	0.9	1.4	2.6	2.9	2.0					
	L		15	15	34	20	20	17					
	C_{in}		97	92	80	82	82	76					
G. S.	V		1.2	2.0	1.4	4.1	5.4	4.7					
	L		15	15	32	28	30	30					
	C_{in}		120	128	125	135	140	140					
R. S.	V		0.8	0.4	0.4	1.0	2.9	3.4	4.0				
	L		16	15	15	20	15	14	19				
	C_{in}		129	132	133	168	167	132	134				
L. B.	V	2.7	2.1	2.2	2.3	4.6	11.1	13.3	15.2	15.2			
	L	17	17	16	20	21	19	15	15	8			
	C_{in}	121	103	103	103	115	134	125	125	125			
I. S.	V	1.7	1.9	1.7	1.4	4.3	7.4	12.7	13.0	12.0			
	L	27	13	33	19	21	14	6	11	9			
	C_{in}	155	140	145	168	151	156	170	157	173			

* = Patient with orthostatic hypotension.
† V = Urine flow (ml. per minute); L = Length of period in minutes; C_{in} = Inulin clearance.

The results are summarized in Table IV. In both the patients and the control subjects, errors occurred in the estimation of glomerular filtration rate as a result of the changing urine flows and associated dead space and "wash out" errors. To enable an evaluation of the data Table V lists the urine flow, length of collection period and inulin clearance for the individual studies. During the collection periods when urine flow was not changing markedly from one period to the next, it was believed that the glomerular filtration rate was accurately determined. A detailed protocol from one of the studies of a patient with orthostatic hypotension is presented in Table VI.

In the patients, the enhanced excretion of sodium might be attributed to the greater and more rapid elevation of the GFR associated with ECF expansion. In the normal persons the moderate increase in sodium excretion could also be correlated with the change of GFR. It does not necessarily follow that the increase in sodium excretion is due to the increase in GFR. It is also possible that tubular function *per se* is subject to nervous influences.

DISCUSSION

A previous study indicated that patients with hypofunction of the autonomic nervous system have an increased sensitivity to vasoactive substances of diverse types, both pressor and depressor (1). For example, vasopressin and norepinephrine caused marked hypertension, and oxytocin and nitroglycerine marked hypotension. The wide spectrum of vasoactive substance to which these patients respond suggests that the increased sensitivity results from the absence of vasoregu-

TABLE VI

*Effect of isotonic sodium chloride infusion on renal function in orthostatic hypotension**

Time	Urine volume	Arterial pressure	C_{In}†	Urine Na conc.	UV_{Na}‡	Urine K conc.	UV_K‡	U_{osm}§	UV_{osm}‖	C_{osm}**	C_{H_2O}††
	ml./min.		*ml./min.*	*mEq./L.*		*mEq./L.*		*mOs./L.*			
9:40	0.7	126/90	56	146	102	17	12	535	374	1.3	−0.6
9:50	0.9	130/90	57	148	134	17	15	505	453	1.6	−0.7
10:00	0.9	128/88	56	165	172	18	17	645	452	1.6	−0.9
10:01	Begin isotonic sodium chloride infusion at 56 ml. per minute										
10:20	3.4	156/96	79	144	488	9	32	353	1,200	4.2	−0.8
10:30	12.8	156/96	96	110	1,410	5	65	245	3,140	11.1	1.8
10:40	25.6	154/100	96	105	2,660	4	90	219	5,610	19.7	5.9
10:56	31.4	150/94	90	111	3,450	3	109	232	7,280	25.5	5.9
10:56	End isotonic sodium chloride infusion										
11:00	36.2	144/90	98	112	4,040	3	126	232	8,400	29.3	6.9
11:10	26.0	138/88	92	105	2,730	4	92	219	5,700	19.9	6.2
11:20	17.5	138/94	74	101	1,770	4	65	213	3,730	13.0	4.5
11:30	10.5	144/94	65	114	1,200	4	49	248	2,605	9.1	1.4
11:50	6.0	144/94	72	168	1,050	8	47	370	2,220	7.8	−1.8
12:00	5.2	144/94	71	176	916	9	45	387	2,010	7.0	−1.8

* Patient B. B. Time: 8:40, Inulin prime; then 20 mg. per minute inulin infusion in 5 per cent glucose at 1 ml per minute.

† Inulin clearance.

‡ Sodium and potassium excretion in microequivalents per minute.

§ Urine osmolality.

‖ Total solute excretion in microosmoles per minute.

** Clearance of total solute.

†† Solute-free water clearance.

latory reflexes. In normal man, these reflexes compensate for changes in arterial pressure from any cause and maintain arterial pressure within narrow homeostatic limits.

Thus, in normal man, expansion of extracellular fluid volume with saline did not result in significant alteration of arterial pressure. In contrast, volume changes in the patients with deficient autonomic reflexes resulted in profound changes in arterial pressure, showing that the patients lack the ability to maintain circulatory homeostasis.

In association with the lability of the arterial pressure, these patients have a labile glomerular filtration rate. Passive tilting from a recumbent to an upright posture resulted in a marked fall in glomerular filtration rate and decreased sodium excretion. On the other hand, rapid expansion of the extracellular fluid volume resulted in an enhanced sodium excretion in these patients when compared to normal persons. In the patients the increased rate of excretion could be attributed to a rapid increase in glomerular filtration rate.

The actual level of the arterial pressure could not be correlated with the change in GFR and sodium excretion. This was demonstrated in the studies in which saline was infused during the hypertensive state associated with methyl fluorohydrocortisone administration: Despite the lack of any further increase in arterial pressure, the GFR and sodium excretion increased markedly.

The hemodynamic and renal responses of these patients to saline administration are analogous to those observed in dogs. The dog has less well-developed autonomic vasoregulatory reflexes than man, consistent with a quadrapedal as compared to a bipedal habitus (5). The dog, as well as other quadrapeds studied, has a labile glomerular filtration rate and consequently is able to excrete rapidly large quantities of exogenously administered salt (6, 7). In normal man, the response of the GFR to intravenously administered salt is less than that observed in the dog. For example, Crawford and Ludemann (8) found that isotonic saline administered at rates as high as 56 ml. per minute increased the GFR by more than 10 per cent in only 4 of 29 subjects studied. In the present study, in none of the control subjects was the magnitude of increase in GFR as great as that observed in the patients. Furthermore, within the control group, the variations in sodium excretion in response to saline administration could be correlated with the change in GFR.

To what is the relative stability of the glomeru-

lar filtration rate in man attributable? It seems possible that the presence of autonomic vasoregulatory reflexes functioning to maintain circulatory homeostasis may result in normal man's relative inability to increase his glomerular filtration rate rapidly and thereby excrete excesses of salt. This hypothesis is supported by the finding that persons with absent vasoregulatory reflexes have a labile filtration rate and an enhanced ability to excrete salt rapidly. Further studies are in progress to test this hypothesis in normal man during ganglionic blockade.

SUMMARY

1. Patients with idiopathic hypofunction of the autonomic nervous system, manifested by postural hypotension, hypohidrosis and impotence, have been found to have an enhanced ability to excrete sodium and solute-free water in response to the rapid infusion of isotonic saline.

2. The augmented solute-free water excretion could be prevented by the administration of vasopressin.

3. The sodium excretion was not prevented by the administration of sodium-retaining adrenal steroids.

4. The enhanced sodium excretion was associated with acute elevation of glomerular filtration rate associated with ECF expansion.

5. In addition, acute expansion or depletion of blood or extracellular fluid volume in these patients resulted in profound changes in arterial pressure, indicating a lack of ability to maintain circulatory homeostasis.

6. The experiments suggest that the presence of well-developed autonomic vasoregulatory reflexes functioning to maintain circulatory homeostasis may result in man's relative inability to rapidly increase his glomerular filtration rate and thereby rapidly excrete excesses of salt.

ACKNOWLEDGMENTS

I would like to acknowledge my indebtedness to Drs. Kendall A. Elsom and Roscoe F. Morton, who referred two of the patients with orthostatic hypotension, and to Drs. Harold Dodge and Eugene Braunwald, who collaborated in several of the studies.

REFERENCES

1. Wagner, H. N., Jr., and Braunwald, E., The pressor effect of the antidiuretic principle of the posterior pituitary in orthostatic hypotension. J. Clin. Invest., 1956, **35**, 1412.
2. Walser, M., Davidson, D. G., and Orloff, J., The renal clearance of alkali-stable inulin. J. Clin. Invest., 1955, **34**, 1520.
3. Liddle, G. W., and Richard, J. E., 2-methyl hydrocortisones: a new series of steroids with enhanced potency and prolonged action. Science, 1956, **123**, 324.
4. Wagner, H. N., Jr., Davidson, D. G., and Orloff, J., The graded effect of purified vasopressin and urinary solute on the excretion of solute-free water. Clin. Research Proc., 1957, **5**, 23.
5. Mayerson, H. S., Orthostatic circulatory failure ("gravity shock") in the dog. Am. J. Physiol., 1944, **141**, 227.
6. Wesson, L. G., Jr., Anslow, W. P., Jr., Raisz, L. G., Bolomey, A. A., and Ladd, M., The effect of sustained expansion of extracellular fluid volume upon filtration rate, renal plasma flow and electrolyte and water excretion in the dog. Am. J. Physiol., 1950, **162**, 677.
7. Dicker, S. E., The action of mersalyl, calomel, and theophylline sodium acetate on the kidney of the rat. Brit. J. Pharm., 1946, **1**, 194.
8. Crawford, B., and Ludemann, H., The renal response to intravenous injection of sodium chloride solutions in man. J. Clin. Invest., 1951, **30**, 1456.

ACID MUCOPOLYSACCHARIDES IN NORMAL SERUM [1]

By ALFRED JAY BOLLET,[2] MARIA W. SERAYDARIAN, AND WILLIAM F. SIMPSON

(From the Medical Service of Wayne State University, College of Medicine and Detroit Receiving Hospital, Detroit, Mich.)

(Submitted for publication March 22, 1957; accepted June 3, 1957)

Mucopolysaccharide which contains uronic acid has recently been described in normal plasma (1, 2). A method for the quantitation of plasma acid mucopolysaccharide was described by Badin, Schubert and Vouras (1), based on the observation that chondroitin sulfate added to plasma is found in euglobulin precipitates (3). These authors reported an increased amount of acid mucopolysaccharide in the plasma of 10 patients with rheumatoid arthritis (1).

The study of acid mucopolysaccharides in blood which are chemically related to those of connective tissue might reveal information of interest concerning the diseases which primarily affect connective tissue. Accordingly, a relatively practical method was devised for measurement of the uronic acid-containing polysaccharide in the presence of large amounts of protein, and studies of the nature of the mucopolysaccharides present in blood were undertaken. Observations made on plasma and serum from normal adults are reported.

METHODS

Quantitation of total uronic acid-containing mucopolysaccharide. The method which was devised for this purpose is based on the method of Badin, Schubert, and Vouras (1), with several modifications. The procedure is as follows:

1. Five 10-ml. samples of plasma or serum were dialyzed for 72 hours at 4° C. against 0.0063 M acid-phosphate buffer of pH 5.8, to precipitate the euglobulins.

2. The contents of the dialysis bag were diluted with 1.5 volumes of citric-phosphate buffer, the precipitate centrifuged, and the supernatant decanted. The precipitate was dissolved in 10 ml. 0.14 M NaCl in 0.02 M NaOH.

3. Three ml. of 1.7 M perchloric acid was then added slowly with constant agitation by a magnetic stirrer. After about 30 minutes, the precipitate was removed by filtration through Whatman No. 5 paper, and a 10-ml. aliquot of the filtrate was dialyzed against running cold tap water overnight.

4. Two-tenths ml. of 1 per cent protamine sulfate was added to the dialyzed material. After 30 minutes in the cold, the precipitate was centrifuged.

5. The precipitate was dissolved in 2.2 ml. of 2 M acetate buffer of pH 5.0 with slight warming, and 1.0-ml. aliquots were used for duplicate uronic acid determinations using the carbazole method of Dische (4). The optical density of each tube was read after heating with sulfuric acid and again 2 hours after adding carbazole; the initial nonspecific color was then subtracted from the final reading.

Paper chromatography. An attempt was made to identify the uronic acid-containing mucopolysaccharides by paper chromatography. The dialyzed material obtained after Step 3 was brought to dryness by lyophilization, dissolved in approximately 0.4 ml. of water, and applied as a spot to Whatman No. 1 paper. Ascending chromatography was done in two solvent systems: 1) ethanol, 52 ml. plus 48 ml. of 0.06 M phosphate buffer of pH 6.6, and 2) propanol, 37 ml. plus 63 ml. of 0.06 M phosphate buffer of pH 6.6, according to the procedure of Kerby (5). After 42 hours, the chromatograms were dried in air, then fixed in 20 per cent formaldehyde in ethanol for 15 minutes, immersed in ether and dried in air. The fixed chromatograms were sprayed with recrystallized toluidine blue solution (40 mg. toluidine blue in 80 ml. acetone and 20 ml. H_2O), rinsed in 2 per cent acetic acid and then distilled water. The staining procedure was a modification of that of Hamerman (6). For chemical determinations, the material obtained from 40 to 80 ml. of normal human plasma was lyophilized and then applied to paper as a six-inch band. After 42 hours, one edge of the paper chromatogram was cut off and stained as a marker, and the corresponding areas containing mucopolysaccharide were eluted from the paper with water. The precipitate obtained following the addition of 0.2 ml. of protamine sulfate to each eluate was divided; part was analyzed for its content of uronic acid as described. An aliquot was taken for the determination of hexosamine by the method of Boas (7). A paper control was eluted and subjected to the same procedure.

Effect of hyaluronidase. The effect of hyaluronidase on the uronic acid containing mucopolysaccharides was determined by adding, to the dialyzed material obtained after Step 3, 200 turbidity reducing units of testicular hyaluronidase (Wyeth Corp.), plus acetate buffer of pH 6.0 and NaCl to a final concentration of 0.1 M acetate

[1] This investigation was supported by grants from the Michigan Chapter of the Arthritis and Rheumatism Foundation and the Research and Education Corporation of Detroit Receiving Hospital.

[2] Markle Scholar in the Medical Sciences.

TABLE I

Recovery of hyaluronic acid and chondroitin sulfate added to 10-ml. samples of normal plasma

Amount of added hyaluronic acid	Amount of added chondroitin sulfate	Recovery
μg.	μg.	*per cent*
43		84.6
43		87.1
43		85.2
65		85.8
65		87.7
108		82.3
108		80.8
54		77.5
	21.2	100.1
	21.2	94.7
	12.4	90.9
	12.4	100.4
	Average	88.1

and 0.15 M NaCl. After incubation at 37° C. for 3 hours, perchloric acid was added, the precipitate removed and the supernatant material dialyzed against tap water for 15 hours. For chromatography, dialyzed material was lyophilized, dissolved in 0.3 ml. of water and applied to paper. For quantitation, the dialyzed material was then treated as in Steps 4 and 5.

Determination of the relative amounts of glucosamine and galactosamine was done by the method of Roseman and Daffner (8) and analyses for hexose were done by the anthrone method of Kapuscinski and Zak (9), using a standard containing equal parts of galactose and mannose.

RESULTS

Evaluation of the quantitative method

In order to determine if the method described above allows the detection of small amounts of acid mucopolysaccharide in the presence of large amounts of protein, varying quantities of hyaluronic acid prepared from cock's comb by the method of Boas (10) and commercial chondroitin sulfate were added to normal plasma. Recoveries of these substances (Table I) varied between 77.5 and 100 per cent, averaging 88.1 per cent.

The absorption spectrum between 420 mμ and 620 mμ after the addition of carbazole to the material obtained from plasma was identical with that of pure glucuronolactone. Protamine gave no color with the reagents used in the uronic acid determination and did not interfere with the color development in the reaction. Replicate determinations done on plasma obtained from normal individuals and carried through the entire procedure

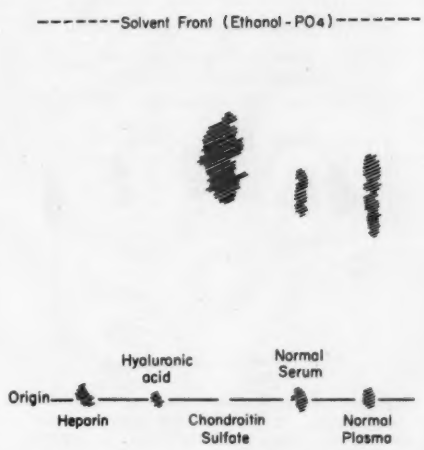


FIG. 1. DIAGRAM OF FILTER PAPER CHROMATOGRAM OF NORMAL PLASMA AND SERUM IN ETHANOL-PHOSPHATE SYSTEM

revealed an error of about 6 to 9 per cent. Anthrone determinations done on the protamine precipitate obtained from plasma by the above procedure revealed the presence of hexose. Although there was insufficient protein to give appreciable color with the sulfuric acid alone, the hexose present gave some color with the carbazole. At 520 mμ, however, hexoses (glucose, galactose and mannose) give relatively little color and it was calculated that the hexose present did not account for more than 10 per cent of the increase in color following addition of carbazole.

Nature of mucopolysaccharides found in normal plasma

The method used established that the material being measured was non-dialyzable, precipitated with the euglobulins under the conditions mentioned, was soluble in perchloric acid and was precipitated by protamine. It was found to contain uronic acid.

Following incubation with hyaluronidase, uronic acid could not be demonstrated in the small amount of precipitate which appeared after addition of protamine.

Filter paper chromatography revealed two components staining metachromatically with toluidine blue. Figure 1 shows the results of paper chromatography in solvent 1 (ethanol-phosphate). Of the polysaccharides tested, only chondroitin

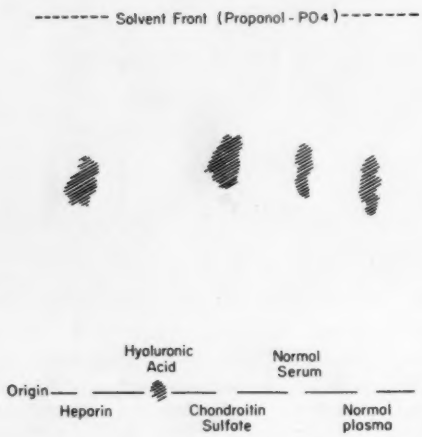


FIG. 2. DIAGRAM OF FILTER PAPER CHROMATOGRAM OF NORMAL PLASMA AND SERUM IN PROPANOL-PHOSPHATE SYSTEM

sulfate (commercial as well as material prepared from bovine nasal septum by the method of Meyer and Smyth [11]) was found to move from the origin in this solvent system. Samples of commercial heparin, β-heparin (chondroitin-sulfate B), and samples of hyaluronic acid (prepared from umbilical cord, cock's comb, vitreous humor and synovial fluid) did not move. The material prepared from plasma was found to give one spot near the solvent front, with an Rf value close to that of chondroitin sulfate (this will henceforth be referred to as component I), and a second spot at the origin.

Figure 2 shows the results of chromatography in solvent 2 (propanol-phosphate). In this solvent system, samples of chondroitin sulfate moved close behind the solvent front, while heparin and β-heparin moved slightly slower. All samples of hyaluronic acid tested remained at the origin. The material obtained from plasma showed no significant amount of staining at the origin, and therefore the component which stayed near the origin in solvent 1 moved in solvent 2; it will henceforth be referred to as component II.

Chromatography after treatment of the material obtained from plasma with hyaluronidase failed to reveal any metachromatic staining material in either solvent system, although some non-metachromatic material could be seen faintly at the origin in ethanol-phosphate and near the solvent front in propanol-phosphate.

Hexosamine to uronic acid molar ratios were determined on the protamine precipitates of Step 4 and of eluates from paper chromatograms. The protamine precipitates of Step 4 were found to have an average hexosamine: uronic acid ratio of 1.3 : 1. The eluates from the ethanol-phosphate system, which separated the two metachromatic components, revealed hexosamine: uronic acid ratios of 1.1 : 1 in the area of component I. The ratio at the origin, corresponding to the area of component II, was 1.5 : 1. These values were calculated after a correction was made for the blank obtained by elution of control samples of paper treated in the same fashion.

The hexosamine in the protamine precipitate of Step 4 was found to be 72 per cent galactosamine and 28 per cent glucosamine.

Level of acid mucopolysaccharide in normal serum

The acid mucopolysaccharide levels of serum from 34 normal adults, obtained at the time of blood donations and from laboratory personnel, revealed uronic acid contents ranging between 215 and 385 μg. per 100 ml. (Table II) measured as glucuronic acid. The mean level was 277 μg. per 100 ml., with a standard deviation of $\pm$ 42 μg. per 100 ml. There was no apparent difference between the levels found in males and females, in the individuals studied. No significant change with age was found in the range between 18 and 52 years.

Serum samples taken after 12 hours fasting and one hour postprandial from six chronically ill hospitalized patients with varying initial levels showed an average fall of 7.5 per cent in the concentration of acid mucopolysaccharide after eating. This was not considered to be significant.

DISCUSSION

The method described for the quantitative determination of uronic acid containing mucopolysaccharide in serum is essentially a modification of the method of Badin, Schubert, and Vouras (1). Considerably less starting material is used, and digestion of euglobulin precipitate for three days with pepsin and then three days with trypsin is

omitted, since it was found that perchloric acid removed a sufficient proportion of protein from the euglobulin solution without loss of uronate-containing polysaccharide. A step involving precipitation in alcohol and ether for 48 hours was also found to be unnecessary. Protamine sulfate is used in place of lyzozyme to precipitate the polysaccharide, since it gives quantitative recoveries of hyaluronic acid, chondroitin sulfate and heparin, and is a simple protein of known amino acid composition which contains no neutral sugar or hexosamine to complicate chemical analyses of the precipitate. Since the protamine does not affect the uronic acid determination, internal standards and correction factors are not necessary.

An attempt was made to avoid dialysis after precipitation with perchloric acid, by removing the perchloric acid with potassium hydroxide. Although the pH of the supernatant was adjusted to that found after dialysis (7.35), quantitative recovery of polysaccharides could not be obtained.

Badin, Schubert, and Vouras (1) found the uronic acid content of the mucopolysaccharide isolated from plasma of seven normal adults by their method to range between 153 and 281 μg. per cent, averaging 206 μg. per cent. This is lower than the range which we found in serum, but one possible explanation is that the extra steps in their method might have resulted in some loss.

The present study does not allow definite conclusions to be drawn as to the nature of the two components found in normal serum. The fact that no uronic acid containing polysaccharide was recovered after treatment with testicular hyaluronidase suggests a relationship to the acid mucopolysaccharides of connective tissue. Component I had chromatographic characteristics resembling chondroitin sulfate prepared from cartilage and presumably could have been chondroitin sulfate A or C, or a mixture of the two. It is of interest that Schiller and Dewey (2) have isolated a component from normal plasma by alcohol precipitation after enzyme digestion and trichloracetic acid precipitation of the proteins and identified it as chondroitin sulfate A (12). Component II had chromatographic characteristics resembling heparin and beta-heparin (chondroitin sulfate B) but differed from those substances in that it was digested by testicular hyaluronidase. Most of the hexosamine in the protamine precipitates was found to be galactosamine, suggesting that both components contain that substance, but further study of the nature of component II will be necessary to establish its identity.

TABLE II

Serum acid mucopolysaccharide levels, measured as uronic acid, in normal adults

Age	Sex	Uronic acid	Age	Sex	Uronic acid
		μg. %			μg. %
18	M	219	30	M	268
20	M	234	30	M	265
20	F	256	31	F	273
22	M	279	31	M	248
23	F	324	31	M	251
23	M	324	34	M	385
23	F	282	35	F	351
25	M	246	37	M	288
26	M	271	38	M	330
26	M	259	41	M	277
26	M	247	42	M	330
26	F	247	42	M	275
27	M	215	42	F	275
27	M	290	44	M	222
27	M	241	48	M	352
28	M	253	51	M	223
28	M	281	52	M	346

In view of the presence in serum of polysaccharides containing components and characteristics found in polysaccharides of connective tissue, determination of the serum levels and of the qualitative nature of the material present in various clinical states might reveal information of interest concerning the diseases which primarily affect connective tissue.

SUMMARY

1. Polysaccharide containing uronic acid and hexosamine can be isolated from 5- to 10-ml. quantities of plasma or serum by a method suitable for quantitation.

2. This material contains two components on filter paper chromatography which stain metachromatically with toluidine blue. The chromatographic characteristics of one resembles that of samples of chondroitin sulfate, and the other resembles heparin and beta-heparin, but both components are labile to testicular hyaluronidase.

3. No material with the chromatographic mobility of hyaluronic acid could be found in samples of normal plasma.

4. Sera of 34 normal adults had an average level of mucopolysaccharide of 277 μg. per cent, measured as glucuronic acid.

ACKNOWLEDGMENTS

Samples of hyaluronic acid prepared from umbilical cord and vitreous humor, of beta heparin, and one of the samples of chondroitin sulfate prepared from bovine nasal cartilage used in this study were kindly supplied by Dr. Saul Roseman. The authors would also like to acknowledge with gratitude the advice and editorial criticism of the staff of the Rackham Arthritis Unit of the University of Michigan, especially Drs. William D. Robinson and Saul Roseman, and of Dr. Richard Winzler of the University of Illinois.

REFERENCES

1. Badin, J., Schubert, M., and Vouras, M., Plasma polysaccharide fraction containing uronic acid, in normal subjects and in patients with rheumatoid arthritis. J. Clin. Invest., 1955, **34**, 1317.
2. Schiller, S., and Dewey, K. F., Isolation of chondroitinsulfuric acid from normal human plasma. Federation Proc., 1956, **15**, 348.
3. Badin, J., and Schubert, M., Conditions of formation of euglobulin-like precipitates from serum proteins and chondroitin sulfate. J. Clin. Invest., 1955, **34**, 1312.
4. Dische, Z., A new specific color reaction of hexuronic acids. J. Biol. Chem., 1947, **167**, 189.
5. Kerby, G. P., The occurrence of acid mucopolysaccharides in human leukocytes and urine. J. Clin. Invest., 1955, **34**, 1738.
6. Hamerman, D., Staining methods in chromatography of acidic and neutral mucopolysaccharides. Science, 1955, **122**, 924.
7. Boas, N. F., Method for the determination of hexosamines in tissues. J. Biol. Chem., 1953, **204**, 553.
8. Roseman, S., and Daffner, I., Colorimetric method for determination of glucosamine and galactosamine. Anal. Chem., 1956, **28**, 1743.
9. Kapuscinski, V., and Zak, B., Use of perchloric acid filtrate and stabilized anthrone for determination of serum glucose. Am. J. Clin. Path., 1953, **23**, 784.
10. Boas, N. F., Isolation of hyaluronic acid from the cock's comb. J. Biol. Chem., 1949, **181**, 573.
11. Meyer, K., and Smyth, E. M., On glycoproteins. VI. The preparation of chondroitinsulfuric acid. J. Biol. Chem., 1937, **119**, 507.
12. Schiller, S., Personal communication.

THYROXINE-BINDING BY SERUM AND URINE PROTEINS IN NEPHROSIS. QUALITATIVE ASPECTS [1]

By JACOB ROBBINS, J. E. RALL, AND MARY L. PETERMANN [2]

(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland; and The Sloan-Kettering Institute for Cancer Research, New York, N. Y.)

(Submitted for publication April 1, 1957; accepted May 9, 1957)

The occurrence of hypercholesterolemia, lowered basal metabolic rate and depressed serum protein-bound iodine in the nephrotic syndrome has directed attention to the thyroid gland in this disorder. Although hypothyroidism has been suspected, these patients are usually considered, on clinical grounds, to be euthyroid, and they fail to respond in a specific manner to even large doses of thyroid hormone (1–3). Since the thyroid gland itself appears to be functioning normally, or even excessively, with respect to iodine accumulation (4), and responds in a normal fashion to thyrotropin and exogenous thyroxine (5), possible disorders in the peripheral metabolism of thyroid hormone have been investigated. Recant and Riggs (5) have suggested that the loss of hormonal iodine in the urine, although considerable, is insufficient to explain the low serum iodine concentration. These investigators demonstrated a normal degradation rate for exogenous thyroxine, whereas Rasmussen (6) found a lower than normal amount degraded each day.

Inasmuch as the circulating thyroid hormone is almost entirely bound to serum protein (7), and since the protein components of serum are extensively altered in nephrosis, a possible defect in this aspect of thyroid physiology has been suspected (5). A preliminary report from our laboratory (8), as well as one by Recant (9), has suggested the presence of a qualitatively abnormal thyroxine-protein complex in nephrotic serum. The data presented in this report, however, indicate that in the nephrotic state, the serum proteins which interact with thyroxine are normal from a physical standpoint.

[1] Presented in part at the Laurentian Hormone Conference, Mont Tremblant, Canada, 1956.

[2] Supported by a grant from the National Cancer Institute of the United States Public Health Service, No. C-471.

MATERIALS AND METHODS

Serum was obtained in the fasting state from 11 patients with the nephrotic syndrome.[3] Urine was obtained from two patients. Clinical and laboratory data for these patients are listed in Table I.

Mixtures of serum (or concentrated urine, *cf.* below) and labeled L-thyroxine [4] were prepared by adding serum to the appropriate quantity of thyroxine previously dried *in vacuo* (10), and were equilibrated for at least 15 hours at 4° C. Radiochemical purity was assessed by chromatography. The thyroxine was repurified, when necessary, to a purity of 90 per cent or greater before addition of serum (11). The final concentration of thyroxine was determined by iodine analysis of the serum (or urine) and of the thyroxine solution, corrected for losses during preparation of the mixture. Iodine analyses were performed by modification of the Barker (12) or Zak, Willard, Myers, and Boyle (13) [5] procedures. A total of 52 serum mixtures and 9 urine mixtures were prepared, in which the concentration of added thyroxine varied from 0.009 to 6.3 μg. per ml.

Zone electrophoresis in horizontally suspended paper strips was carried out by the conventional or reverse-flow technique (11) in barbital buffer, pH 8.6, ionic strength 0.1. Radioactivity on the paper strips was measured with a continuously-recording, counting rate meter and an end-window Geiger-Mueller tube. The width of the counting aperture was ⅛ inch or ¼ inch.

Moving boundary electrophoresis in acetate-chloride buffer at pH 4.5 was carried out as described earlier (14), except that a lower voltage (about 4 volts per cm.) and a longer time (about 8 hours) were used to avoid convective disturbances in the lipoprotein boundaries. Ultracentrifugation, employing a partition cell, was performed according to techniques reported elsewhere (15).

In one patient (Case No. 7), 22 μg. of L-thyroxine labeled with 1.0 mc. of I^{131} and dissolved in 50 per cent

[3] These sera were generously supplied by Dr. Lee Farr from patients hospitalized at the Brookhaven National Laboratory, Upton, New York, and by Drs. Hans Keitel, James Baxter and Howard Goodman from patients in the National Heart Institute, National Institutes of Health, Bethesda, Maryland.

[4] L-thyroxine labeled with I^{131} was obtained from Abbott Laboratories, Oak Ridge, Tennessee.

[5] Performed by J. Benotti, Boston Medical Laboratory, Boston, Mass.

TABLE I

Clinical and laboratory data on patients with the nephrotic syndrome

Case no.	Age	Sex	Diagnosis	Blood urea N	Serum levels			
					Total protein	Albumin	Cholesterol	Protein-bound iodine
	yrs.			*mg. %*	*Gm. %*	*Gm. %*	*mg. %*	*μg. %*
1	4	M	Idiopathic	25	5.0	1.1		0.4
2	4	M	Idiopathic	10	4.5	2.0	491	5.4†
3	19	M	Hodgkin's disease	6.3	6.1		535	1.5
4A*	3	F	Idiopathic	5.7	4.3	1.2	959	1.7
B					4.0	0.9	785	
5	6	F	Idiopathic	200	3.9	2.1	676	
6	6	F	Idiopathic	20	4.1	0.6	1,522	1.2
7	23	F	Disseminated lupus erythematosis‡	36	3.6	1.2	510	5.2
8A*	54	M	Primary amyloidosis‡	38	4.1	0.8	807	2.1
B				39	4.0	2.1	595	1.4
9	7	M	Idiopathic‡	16	5.4	1.7	814	3.2
10	35	F	Disseminated lupus erythematosis‡	65	2.7	0.4	493	2.2
11	7	F	Idiopathic	31	3.9	0.4	1,085	0.1

* "A" and "B" refer to sera obtained at different times from these patients.
† Receiving Lugol's solution.
‡ Receiving metacorten.

propylene glycol was injected intravenously. Serum and urine specimens were collected at intervals following the injection. In addition to electrophoretic analysis, the urinary radioiodine was subjected to analysis by the following procedures in a manner similar to that employed with serum: paper chromatography with n-butanol: dioxane (4:1) equilibrated with 2N NH_4OH, extraction with n-butanol (16), salting out with phosphate buffer (17), and dialysis against large volumes of 0.85 per cent NaCl. Prior to electrophoresis, the urinary proteins were concentrated three- to six-fold by dialysis against a 25 per cent solution of polyvinyl pyrrolidone in barbital buffer. This patient's serum and urine were also used for *in vitro* thyroxine experiments.

RESULTS AND DISCUSSION

The results with the thyroxine-serum mixtures may be considered in two categories: those in which most of the thyroxine is associated with the specific thyroxine-binding alpha globulin (TBP), and those in which TBP is saturated, so that secondary thyroxine-binding proteins may be examined. Although it will be evident that these categories overlap, they are, for convenience, considered separately in parts I and II of this section (*cf.* comments).

I. Low thyroxine concentration in serum

Mixtures of labeled L-thyroxine and nephrotic serum were prepared, in which the concentration of added thyroxine was from 0.009 to 0.1 μg. per ml. and in which the total thyroxine concentration (*i.e.*, added thyroxine plus endogenous thyroxine [6]) ranged from 0.015 to 0.18 μg. per ml. The radioactive thyroxine-protein complex was then characterized by zone electrophoresis at pH 8.6, moving boundary electrophoresis at pH 4.5, and ultracentrifugation.

A. Electrophoresis at pH 8.6

The findings presented in Figure 1 are illustrative of the results in 10 nephrotic subjects. In both conventional and reverse-flow zone electrophoresis, a radioiodine peak which did not coincide exactly with any of the major protein bands accounted for the major portion of the radioiodine in the serum. (A similarly located radioiodine peak was presented in the sera of the remaining patient [Case No. 11], but less than half of the radioiodine was in this zone at the lowest thyroxine level studied, 0.14 μg. per ml.) This peak had a mobility which was of the order of that of the alpha globulins of normal serum. On the basis of direct comparison between nephrotic and normal sera containing thyroxine-I^{131} and run simultaneously, the electrophoretic mobility of the thyroxine-

[6] For the purposes of the calculation, the serum iodine was assumed to consist entirely of thyroxine.

binding protein (TBP) appeared to be the same in both—*i.e.*, intermediate between the alpha-1 and alpha-2 globulins (6, 18–20).

The serum mixtures also contained thyroxine in the albumin zone. In conventional zone electrophoresis this comprised from 1 to 8 per cent of the total serum thyroxine, whereas in the reverse-flow experiments it comprised from 18 to 33 per cent (see part II). In addition, all of the sera contained a third radioiodine peak which moved just ahead of, and was poorly separated from the albumin peak (Figure 1). In most sera, this component was rather small (2 to 7 per cent of total serum thyroxine) and was similar to the "pre-albumin" radioiodine component noted in normal serum (10). In three subjects (Case Nos. 8B, 9, and 10), however, this component was more prominent, particularly in the reverse-flow experiments, and comprised approximately 10 to 25 per cent of the total serum radioiodine. The pattern in Figure 1C. contained 17 per cent of its radioiodine in this component. It is uncertain whether or not this "pre-albumin" component is the same as that which is found in cerebrospinal fluid (21, 22), where it has been shown to form a complex with thyroxine (16, 23). The pre-albumin component in nephrotic serum was usually less clearly separated from albumin than in cerebrospinal fluid, and its significance is not clear.

The variable quantities of radioiodine which were deposited between the TBP area and the point of application in conventional electrophoresis (and which were often more striking than illustrated in Figure 1) were absent from the reverse-flow experiments, and appeared to be an artifact (see part II). The findings in the patient injected intravenously with labeled L-thyroxine (Case No. 7) were similar to those presented in Figure 1, except that there was less radioactivity in the pre-albumin zone.

B. Electrophoresis at pH 4.5

A mixture of labeled L-thyroxine and serum from one patient (Case No. 4B), containing a total of 0.047 μg. of thyroxine per ml., was subjected to moving boundary electrophoresis at pH 4.5. The results are presented in Figure 2, and may be compared with those for normal sera published earlier (14). The small protein peak in fractions 2 and 3 contained a major radioiodine component and had the highest I^{131}: protein ratio. Inasmuch as the location of albumin in the schlieren pattern could not be determined by inspection, as may be done in the case of normal serum, the fractions denoted in Figure 2 were concentrated (by dialysis against 25 per cent polyvinyl pyrrolidone) and examined by zone electrophoresis at pH 8.6. The protein patterns for fractions 2 and 4 are presented in Figure 2. Whereas the latter contained a protein component with the mobility of albumin, fraction 2 contained a single visible component, the mobility of which was comparable to that of alpha-2 globulin. Since the most acid fraction of albumin is isoelectric at about pH 4.5, it may be concluded that TBP in this nephrotic serum had an isoelectric point slightly lower than pH 4.5. The behavior of this protein was, therefore, indistinguishable from that of TBP in normal sera by electrophoresis at both alkaline and acid pH.

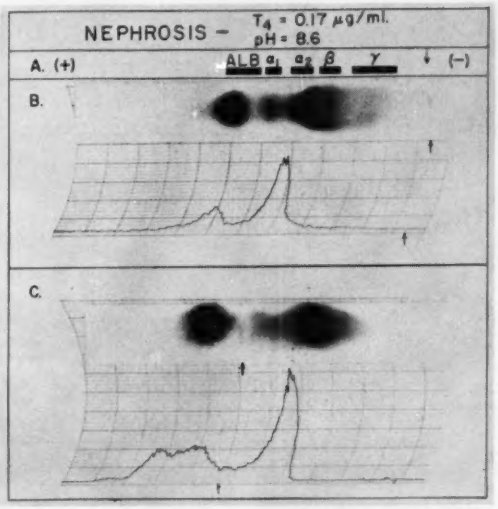


FIG. 1. ZONE ELECTROPHORESIS OF A MIXTURE OF LABELED L-THYROXINE AND SERUM FROM CASE NO. 8B, LOW THYROXINE CONCENTRATION

A. and B., conventional method. C., reverse-flow method. In B. and C., the paper strips, stained with bromphenol blue, are aligned with records of their radioactivity. The arrows indicate the points at which serum was applied. The distances between base-line radioactivity and the bottom lines of the charts represent background counting rates. The bars in A. indicate the location of protein zones in a normal serum run simultaneously with B. T_4 = total serum thyroxine.

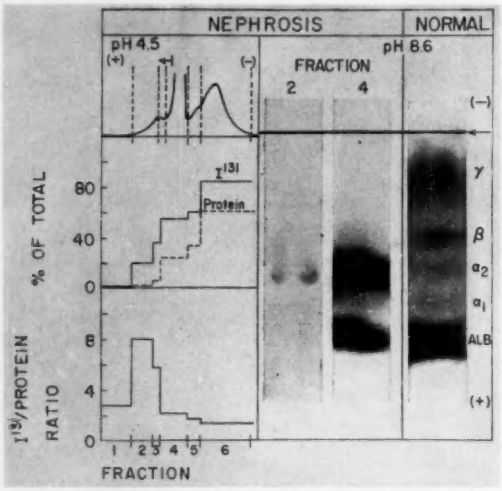


FIG. 2. MOVING BOUNDARY ELECTROPHORESIS OF A MIXTURE OF LABELED L-THYROXINE AND SERUM FROM CASE NO. 4B, TOTAL THYROXINE CONCENTRATION = 0.047 μG. PER ML. (THE TBP AREA IN CONVENTIONAL ZONE ELECTROPHORESIS AT PH 8.6 CONTAINED 71 PER CENT OF THE THYROXINE)

Top left: tracing of schlieren diagram in acetate-chloride buffer. The top of the largest peak is cut off. The vertical line at the top indicates the starting boundary, and the arrow points in the ascending direction. The broken lines indicate the fractions taken for analysis. Middle left: radioiodine and protein in the fractions, the latter measured by ultraviolet absorption at 275 mμ. Bottom left: radioiodine-protein ratios in the fractions. Right: zone electrophoretic patterns in barbital buffer of concentrated fractions 2 and 4 compared with that of normal serum. The strips are stained with bromphenol blue.

C. *Ultracentrifugation*

The thyroxine-protein complex in two sera, (Case Nos. 1 and 4B) was studied in the ultracentrifuge partition cell. In one (Case No. 1), the serum was first subjected to centrifugation in the preparative ultracentrifuge (untreated whole serum at 105,000 × G for 2½ hours at 8° C.) and the low density lipids which floated to the top were removed. This low density material contained no thyroxine, and zone electrophoresis of the remaining serum revealed a radioactivity pattern identical with that in whole serum.

The results of the partition cell experiments are presented in Figure 3 and Table IIA. The rate of disappearance of radioiodine from the top compartment of the cell (Figure 3) was similar to

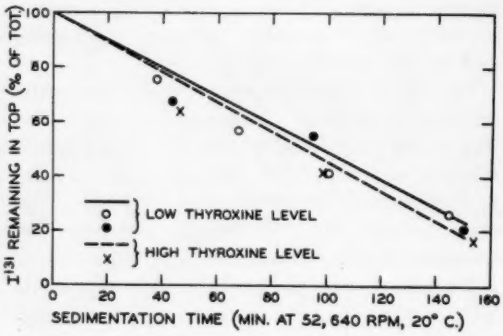


FIG. 3. SEDIMENTATION IN THE ULTRACENTRIFUGE PARTITION CELL OF MIXTURES OF LABELED L-THYROXINE AND SERUM

The lines are theoretical curves, calculated from the sedimentation equations, where s_{20} for TBP in diluted serum = 3.22 S, s_{20} for albumin = 4.05 S, and s_{20} for iodide = 0. The symbols represent experimental data, each point being the result of a separate experiment. Pertinent data are as follows:

Symbol	Case no.	Total thyroxine	TBP	Albumin	Iodide
		μg./ml.		total I^{131} %	
——			70	25	5
○	1	0.044	66	29	5
●	4 B	0.047	71	24	5
- - -			40	55	5
X	1	0.28	39	56	5

Since the proportion of I^{131} with TBP and albumin were determined by conventional zone electrophoresis, it is probable, especially at the high thyroxine level, that TBP was overestimated and albumin underestimated. This error does not significantly affect the results of the ultracentrifuge experiments. Any radioiodine present in the alpha-2, beta, or gamma globulin areas was considered to be derived from thyroxine associated with albumin. In the instance of Case No. 1, 0.28 μg. per ml., the "albumin" radioiodine was composed of 32 per cent of total I^{131} in the alpha-2 globulin zone, 16 per cent in the beta and gamma globulin zones, and only 8 per cent in the albumin zone. Iodide-I^{131} was determined by chromotography.

that expected with normal serum containing a similar distribution of thyroxine between TBP, albumin and iodide. The sedimentation coefficient of TBP (Table IIA) was calculated from the partition cell equations (15) by using the value $s_{20,w} = 4.2$ S [7] for albumin and the distributions of radioiodine between TBP, albumin and iodide as

[7] $s_{20,w} = s_{20} \times 1.037$.

given in the legend to Figure 3. The drift toward a lower sedimentation coefficient with increasing centrifugation time suggests that these sera may have contained a small amount of a rapidly sedimenting, radioiodine component.

One of these sera (Case No. 1) was examined optically in the analytical ultracentrifuge and was found to contain a rapidly sedimenting component ($s_{20,w} = 17.1$ S) which contained 16.5 per cent of the total protein. When serum proteins from this patient were separated by moving boundary electrophoresis in barbital buffer, pH 8.6, and samples removed from the descending limb of the cell examined in the analytical ultracentrifuge, the rapidly sedimenting component was found to be associated with the large alpha-2 globulin peak. This finding is consistent with recent studies by Brown and co-workers (24, 25) who have shown that a macroglobulin, which is probably an alpha glycoprotein, is present in normal serum and is markedly increased in nephrosis. The association of approximately 5 per cent of the serum thyroxine with such a macroglobulin could produce the drift seen in Table II. The sedimentation coefficients calculated for TBP at the longer time intervals (> 67 minutes), however, would not be affected by the presence of such a rapidly sedimenting component, and, therefore, may represent the true sedimentation rate for TBP in nephrosis. These values for $s_{20,w}$ have a mean of 3.6 S, which is comparable within experimental error to the value of 3.3 S for TBP in normal serum (15). The possible presence of a pre-albumin thyroxine-binding component in these sera (see above) was not considered in the calculations. This omission may not introduce a significant error, inasmuch as the pre-albumin component present in normal serum has a sedimentation rate of the order of serum albumin (26).

TABLE II

Sedimentation coefficients calculated from ultracentrifuge partition cell experiments (cf. legend to Figure 3 for other pertinent data)

Case no.	Time*	$s_{20,w}$ TBP	$s_{20,w}$ Secondary binding protein
	A. Low thyroxine concentration		
	min.	Svedbergs	
1	43.6	5.5	
	95.6	3.2	
	150.3	3.9	
4 B	37.7	4.6	
	67.7	4.4	
	100.4	4.1	
	144.8	3.4	
	B. High thyroxine concentration		
	min.	Svedbergs	
1	45.8	6.5	
	99.5	4.9	
	153.5	4.5	

* Sedimentation time at 52,640 rpm, 20° C.

II. High thyroxine concentrations in serum

Mixtures of labeled L-thyroxine and serum were prepared which contained from 0.2 to 6.3 μg. of thyroxine per ml., amounts sufficient to saturate the thyroxine-binding sites on TBP.

A. Electrophoresis at pH 8.6

The electrophoretic distribution of thyroxine presented in Figure 4A. is representative of the findings in all sera examined at high thyroxine

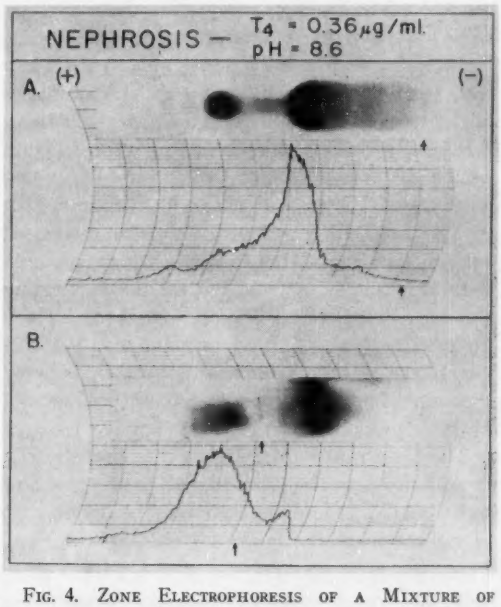


FIG. 4. ZONE ELECTROPHORESIS OF A MIXTURE OF LABELED L-THYROXINE AND SERUM FROM CASE NO. 11, HIGH THYROXINE CONCENTRATION

A., conventional method. B., reverse-flow method. (*Cf.* legend to Figure 1.)

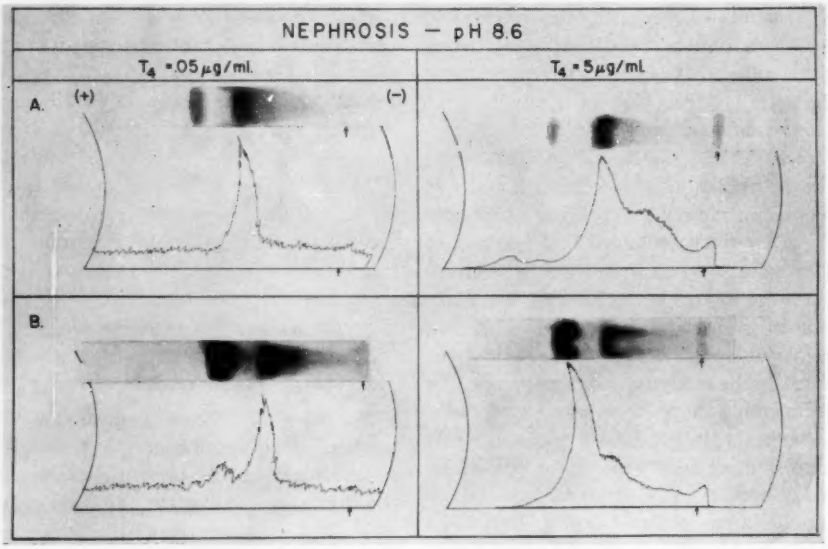


FIG. 5. ZONE ELECTROPHORESIS (CONVENTIONAL METHOD) OF MIXTURES OF LABELED L-THYROXINE AND SERUM FROM CASE NO. 6 AT LOW AND HIGH THYROXINE CONCENTRATIONS

A., no addition. B., human serum albumin added (3.5 Gm. per 100 ml. of serum). (*Cf.* legend to Figure 1.)

concentrations by the conventional technique. A large, broad peak of radioactivity was always found in the region of the dense, abnormal protein zone which had a mobility close to alpha-2 globulin. Although this radiothyroxine peak sometimes coincided with the protein zone, it not infrequently moved somewhat slower or, as in Figure 4A., somewhat faster. Its mobility was distinct from that of TBP. The proportion of thyroxine associated with this zone appeared to vary with the albumin content of the serum. When the serum albumin was high, less thyroxine was found in the alpha-2 globulin region and more in albumin. Variable quantities of radioiodine were present between the point of application and the major peak as well as in the pre-albumin region (see part I).

The pattern in Figure 4B. on the other hand, is typical of the results obtained with the reverse flow technique. In these experiments, the major zone of radioactivity was in the albumin area, a small thyroxine peak was present in the TBP region, and there was no radioactivity in the region of the globulins with mobilities slower than TBP. It is evident from a comparison of Figures 4A. and 4B. that the large alpha globulin component in Figure 4A. could represent an artifact in the conventional electrophoresis procedure. A similar, but quantitatively smaller, artifact has been described in normal serum (11), and appears to be the result of adsorption and dissociation of the thyroxine-albumin complex during migration through the paper strip. In the nephrotic sera, this artifact appeared to be accentuated as a result of the lower albumin concentrations. Figure 5 illustrates the effect of adding human serum albumin [8] to nephrotic serum. The artifact could be reduced by this addition.

That the large magnitude of this artifact is not a characteristic only of nephrotic sera is demonstrated in Figure 6. In this experiment, a mixture of a normal serum and thyroxine, in which the thyroxine-sites on TBP were saturated, was applied to the paper strips in varying amounts. When the volume was small, and the conventional electrophoretic technique was employed, the albumin-bound thyroxine was deposited largely in

[8] Fraction V of human plasma, obtained through the courtesy of Dr. S. T. Gibson and Dr. J. N. Ashworth of the American National Red Cross and E. R. Squibb and Sons, respectively.

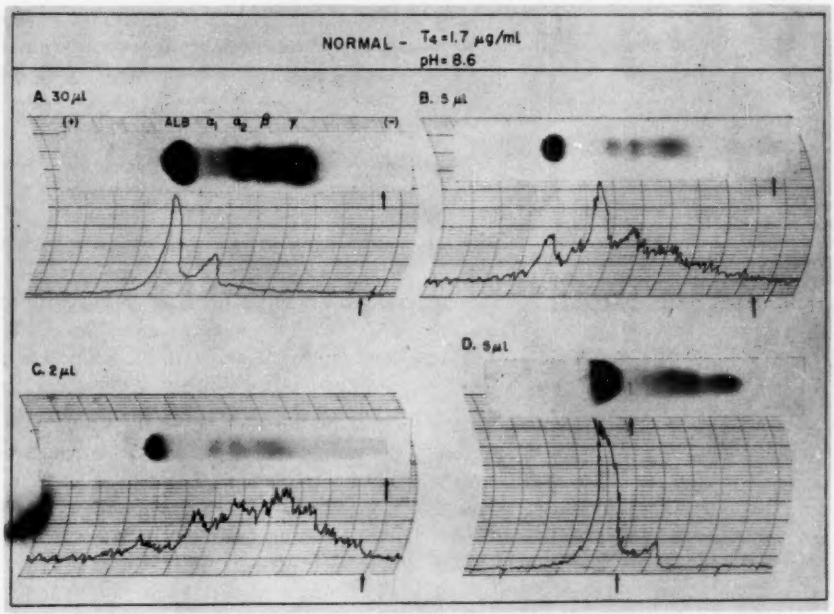


FIG. 6. ZONE ELECTROPHORESIS OF A SINGLE MIXTURE OF LABELED L-THYROXINE AND NORMAL SERUM

A., B., C., conventional method. D., reverse-flow method. The volumes indicate the quantity of serum applied to the paper in each instance. The distribution of I^{131} shown in D. was the same at all volumes with the reverse-flow method. (*Cf.* legend to Figure 1.)

the serum globulin regions. This was apparently a consequence of the reduced amount of albumin on the strip. The failure to find a single large thyroxine peak in the alpha-2 globulin region in this experiment, compared to nephrotic sera at high thyroxine levels, may be due to the relative difference in globulin concentrations in the normal and nephrotic sera. For example, the presence of a globulin in relatively large amount might minimize the adsorption of albumin-thyroxine on the paper up to the point where this globulin itself is deposited.

B. Ultracentrifugation

The possibility that not all the thyroxine at these high concentrations was associated with protein was examined in two sera (Case Nos. 1 and 4A), which contained 1.5 and 6.3 µg. of thyroxine per ml., respectively. The mixtures were subjected to ultracentrifugation in the partition cell at 52,640 rpm for 4 hours. The proportions of thyroxine remaining in the top compartment were 11 per cent and 8 per cent, respectively, indicating that most of the thyroxine was bound to protein even at these high concentrations. In addition, it may be calculated from equations for the interaction of thyroxine with serum protein (16) that even with an albumin level of 1.0 Gm. per cent and 10 µg. of thyroxine per ml., no more than about 2 per cent of the thyroxine would be unbound.

In a single experiment (Case No. 1) similar to that at a low thyroxine level, serum was subjected to ultracentrifugation in the preparative ultracentrifuge at 105,000 × G for 2½ hours. It was found that the serum thyroxine was not associated with the low density lipids.

In one instance (Case No. 1), a thyroxine-serum mixture containing 0.28 µg. of thyroxine per ml. was subjected to ultracentrifugation in the partition cell. The thyroxine-sites on TBP were saturated, and zone electrophoresis by the conventional method revealed only 39 per cent of the radioiodine in the TBP region (see legend to Figure 3). The results are presented in Figure 3 and Table IIB.

TABLE III

*Urine radioiodine analyses after intravenous thyroxine-I^{131} in nephrosis**

Interval after dose	Chromotography†			Solubility in		Dialyzability
	Thyroxine	Iodide	Origin	3.15 M phosphate buffer‡	n-butanol (washed with 4 N NaOH-5% Na_2CO_3)	
0–6 hrs.	88	10	3	10	80	7
6–12 hrs.	96	4	0	10	83	12
12–24 hrs.	91	9	0	17	81	11
1–2 days	78	13	9	15	72	15
2–3 days	78	19	4		68	24

* All values are per cent of the total I^{131} in urine.
† n-butanol: dioxane: ammonia.
‡ Equimolecular $KH_2PO_4 - K_2HPO_4$.

It may be seen in Figure 3 that the rate of disappearance of radioiodine from the upper compartment of the cell was not much different from the theoretical normal curve with a similar thyroxine distribution. This suggested that the sedimentation rate of the protein other than TBP which was interacting with thyroxine was not much different from that for TBP ($s_{20,w} = 3.3$ S) and albumin ($s_{20,w} = 4.2$ S). If it is assumed that this serum contained only two types of protein which could bind thyroxine, one of which was TBP, the sedimentation coefficient of the second protein could be calculated from the sedimentation equations (15). The mean value obtained for the second protein was $s_{20,w} = 4.7$ S, when the sedimentation time was longer than 67 minutes. This value is not significantly different from the sedimentation coefficient of serum albumin. The possible presence of a pre-albumin thyroxine component was again ignored in the calculations (see part IC).

III. Interaction of thyroxine with urine proteins

Urine collected from Patient No. 7 at varying intervals after the intravenous administration of radiothyroxine was analyzed by the procedures listed in Table III. Most of the radioiodine in these urine specimens was in the form of thyroxine bound to protein. It should be noted that this patient had uremia, and there were considerable losses of radioactivity in the feces and by emesis. The latter was shown by chromatography to be in the form of iodide.

The zone electrophoretic analysis of urine concentrated five-fold, presented in Figure 7, is typical of the findings on all the specimens. Most of the radioactivity was in a zone which had a mobility intermediate between alpha-1 and alpha-2 globulin, and a very small amount was associated with albumin.

In two patients (Case Nos. 7 and 10), urine which was concentrated three-fold and which contained no endogenous radioiodine was mixed with labeled thyroxine so that the total thyroxine concentrations varied from 0.11 to 4.3 μg. per ml. The electrophoretic distribution of thyroxine in the urine from Patient No. 7 at thyroxine concentrations lower than 0.2 μg. per ml. was similar to that illustrated in Figure 7. At higher concentrations (analyzed by the reverse-flow method) less radioiodine was present in the "inter-alpha" zone, and correspondingly more in albumin.

In the case of Patient No. 10, however, thyroxine was found only in the albumin zone (in reverse-flow electrophoresis) at even the lowest thyroxine level (0.11 μg. per ml.). This patient's serum contained TBP with the characteristic "inter-alpha" mobility. The reason for the discrepancy between the findings in these two patients is not apparent.

COMMENTS

The results which have been described indicate that the proteins in serum which interact with thyroxine are, in all likelihood, the same in patients with nephrosis as in normal subjects. The presence of subtle differences, however, has not been eliminated by the rather gross methods employed. In addition, the tacit assumption that the thyroxine-binding alpha globulin, TBP, is a single

protein species requires verification, inasmuch as this protein has not yet been isolated.

This report has not dealt with the possibility that quantitative differences in thyroxine-binding may exist. Preliminary data on this question (16) indicate that the thyroxine-binding capacity of TBP (which probably reflects the concentration of TBP) may be lower than normal in nephrosis.[9] With a reduction in specific binding sites, it follows that the concentration of unbound, or free, thyroxine at any level of PBI would be higher than if TBP concentration were normal. Attempts at calculation of the free thyroxine concentration (16) suggest that this value in nephrosis may be normal or even elevated in the presence of a very low PBI. Since there is evidence suggesting that the physiological effects of thyroxine may be a function of the free rather than the total thyroxine (16), euthyroidism and a low PBI could thus coexist in nephrosis. Recant and Riggs (5) reasoned similarly on theoretical grounds. Other features of the complicated thyroid physiology in nephrosis have been discussed elsewhere (5, 6, 16).

It should be pointed out that the thyroxine-binding capacity of TBP, which was quantitated in some of the patients in this study, varied from very low to normal values. This fact is responsible for the wide range of thyroxine concentrations which could be considered as "low thyroxine concentration" in part I of the section on results. The arbitrary division into "low" and "high" concentration groups was based on whether the major portion of the serum thyroxine was complexed with TBP or with the secondary binding protein. Since actual saturation of TBP with thyroxine is approached asymptotically (11, 16), the term "saturation" as used in this report is an approximation and is employed in a descriptive sense.

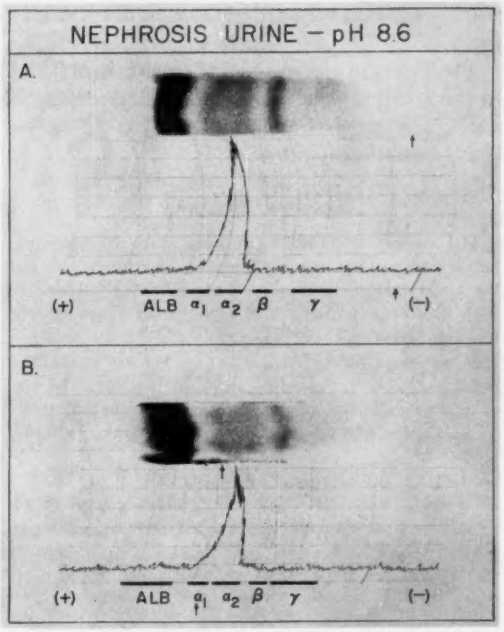


FIG. 7. ZONE ELECTROPHORESIS OF URINE, CONCENTRATED FIVE-FOLD, FROM CASE NO. 7, COLLECTED BETWEEN 12 AND 24 HOURS FOLLOWING INTRAVENOUS INJECTION OF LABELED L-THYROXINE

A., conventional method. B., reverse flow-method. (*Cf.* legend to Figure 1.)

SUMMARY

Sera from 11 patients with the nephrotic syndrome were studied with regard to the nature of the proteins which interact with thyroxine. The proteins were labeled by the addition of radiothyroxine *in vitro* or, in one patient, by intravenous injection. As in normal serum, thyroxine appeared to interact chiefly with two proteins: albumin and an alpha globulin (TBP). There was suggestive evidence that thyroxine interacted to a lesser degree with a third protein component having an electrophoretic mobility at pH 8.6 slightly faster than albumin, and possibly with a fourth component characterized by a rapid sedimentation rate. TBP in the nephrotic sera appeared to be identical with normal TBP with respect to electrophoretic mobility at pH 8.6 and pH 4.5, and sedimentation rate in the ultracentrifuge.

At low thyroxine concentrations, the thyroxine-TBP complex accounted for most of the serum thyroxine. At high thyroxine concentrations, TBP was saturated with thyroxine and the excess thyroxine became associated with albumin. In the presence of low serum albumin concentration, however, conventional zone electrophoresis in filter paper gave rise to an artifact due to adsorp-

[9] Earlier studies (8) which failed to demonstrate a depression of TBP were in error due to the artifact inherent in conventional zone electrophoresis.

tion of albumin-bound thyroxine in the globulin zones.

The urine in one nephrotic patient was found to contain thyroxine in combination chiefly with TBP, and excess thyroxine added to this urine became associated with albumin.

ACKNOWLEDGMENT

The authors wish to thank Mrs. Mary G. Hamilton for performing the moving-boundary electrophoresis and the ultracentrifuge analyses. They are also indebted to Miss Dorothea Jermany and Miss Helen Hagopian for technical assistance.

REFERENCES

1. Epstein, A. A., Thyroid therapy and thyroid tolerance in chronic nephrosis. J. A. M. A., 1926, **87**, 913.
2. Leiter, L., Nephrosis. Medicine, 1931, **10**, 135.
3. Peters, J. P., and Man, E. B., The relation of albumin to precipitable iodine of serum. J. Clin. Invest., 1948, **27**, 397.
4. Skanse, B., Radioactive iodine in the diagnosis of thyroid disease. Acta. med. Scandinav., 1949, Suppl., 235.
5. Recant, L., and Riggs, D. S., Thyroid function in nephrosis. J. Clin. Invest., 1952, **31**, 789.
6. Rasmussen, H., Thyroxine metabolism in the nephrotic syndrome. J. Clin. Invest., 1956, **35**, 792.
7. Trevorrow, V., Studies on the nature of the iodine in blood. J. Biol. Chem., 1939, **127**, 737.
8. Robbins, J., Rall, J. E., and Petermann, M. L., Protein-binding of thyroxine in normal and nephrotic serum (abstract). J. Clin. Invest., 1954, **33**, 959.
9. Recant, L., An abnormality in thyroxine binding in nephrosis (abstract). J. Clin. Invest., 1956, **35**, 730.
10. Robbins, J., and Rall, J. E., Thyroxine-binding capacity of serum in normal man. J. Clin. Invest., 1955, **34**, 1324.
11. Robbins, J., Reverse-flow zone electrophoresis. A method for determining the thyroxine-binding capacity of serum proteins. Arch. Biochem. & Biophys., 1956, **63**, 461.
12. Barker, S. B., Determination of protein-bound iodine. J. Biol. Chem., 1948, **173**, 715.
13. Zak, B., Willard, H. H., Myers, G. B., and Boyle, A. J., Chloric acid method for determination of protein-bound iodine. Anal. Chem., 1952, **24**, 1345.
14. Robbins, J., Petermann, M. L., and Rall, J. E., Electrophoresis of the thyroxine-binding protein of serum at pH 4.5. J. Biol. Chem., 1955, **212**, 403.
15. Petermann, M. L., Robbins, J., and Hamilton, M. G., Sedimentation of the thyroxine-binding protein of serum in the partition cell. J. Biol. Chem., 1954, **208**, 369.
16. Robbins, J., and Rall, J. E., The interaction of thyroid hormones and protein in biological fluids *in* Recent Progress in Hormone Research. New York, Academic Press Inc., 1957, vol. 13.
17. Robbins, J., Thyroglobulin in serum after I^{131} therapy. I. Salting out. J. Biol. Chem., 1954, **208**, 377.
18. Gordon, A. H., Gross, J., O'Connor, D., and Pitt-Rivers, R., Nature of the circulating thyroid hormone-plasma protein complex. Nature, 1952, **169**, 19.
19. Larson, F., Deiss, W. P., and Albright, E. C., Localization of protein-bound radioactive iodine by filter paper electrophoresis. Science, 1952, **115**, 626.
20. Robbins, J., and Rall, J. E., Zone electrophoresis in filter paper of serum I^{131} after radioiodide administration. Proc. Soc. Exper. Biol. & Med., 1952, **81**, 530.
21. Bücher, T., Matzelt, D., and Pette, D., Papierelektropherogramm der Eiweisskörper des Liquor cerebrospinalis. Naturwiss, 1952, **39**, 114.
22. Fisk, A. A., Chanutin, A., and Klingman, W. O., Observations on a rapidly migrating electrophoretic component of cerebrospinal fluid. Proc. Soc. Exper. Biol. & Med., 1951, **78**, 1.
23. Alpers, J. B., and Rall, J. E., The metabolism of iodine in cerebrospinal fluid. J. Clin. Endocrinol. & Metab., 1955, **15**, 1482.
24. Brown, R. K., Baker, W. H., Peterkofsky, A., and Kauffman, D. L., Crystallization and properties of a glycoprotein isolated from human plasma. J. Am. Chem. Soc., 1954, **76**, 4244.
25. Peterkofsky, A., Levine, L., and Brown, R. K., Quantitative estimation of antigens by complement fixation. Studies on the heat-labile alpha-2 glycoprotein. J. Immunol., 1956, **76**, 237.
26. Hoch, H., and Chanutin, A., Electrophoretic studies on fast moving components of human serum. J. Biol. Chem., 1953, **200**, 241.

A COMPARISON OF THE PHENYLALANINE CONTENT OF THE HEMOGLOBIN OF NORMAL AND PHENYLKETONURIC INDIVIDUALS: DETERMINATION BY ION EXCHANGE CHROMATOGRAPHY [1]

By DAVID W. ALLEN [2] and W. A. SCHROEDER

(From the Gates and Crellin Laboratories of Chemistry, California Institutes of Technology, Pasadena, Cal.)

(Submitted March 22, 1957; accepted April 18, 1957)

INTRODUCTION

Despite extensive clinical and biochemical research, the relationship of the metabolic error in phenylketonuria to the clinical symptomatology is unknown (1, 2). The specific biochemical defect, inherited as an autosomal recessive gene, is the lack of phenylalanine oxidase in the liver (3, 4). This lack prevents the effective conversion of phenylalanine to tyrosine and results in high blood levels of phenylalanine and high concentrations in the urine of such metabolites of phenylalanine as phenylpyruvic acid, phenyllactic acid, and phenylacetic acid. How this biochemical defect produces mental deficiency is not known, but the apparent improvement in mental status of patients on low phenylalanine diets suggests a toxic action either of phenylalanine or of its derivatives (5, 6). One possible mode of phenylalanine toxicity would be through increased incorporation of phenylalanine in body proteins and consequent interference with the proper function of these molecules (7). The phenylalanine contents of body proteins of normal and phenylketonuric individuals have been compared but the results are equivocal. Block, Jervis, Bolling, and Webb (8), in the only properly controlled set of experiments, found no significant differences in the phenylalanine content of the serum proteins, erythrocytes, brains, livers, and kidneys of normal and phenylketonuric individuals. Schrappe (9) has claimed that blood proteins from phenylketonurics contain higher than normal amounts of phenylalanine (8.53 per cent) but Keup (10) was unable to confirm this finding.

None of these experiments was performed with sufficient accuracy to detect a difference of one or two phenylalanyl residues per protein molecule. Such a difference might influence the function of a protein if, for example, the phenylalanine replaced a tyrosyl residue. Hence, it was desirable to repeat these experiments with the necessary precision and accuracy so that a difference of only a single phenylalanyl residue might be detected. To achieve this end, a pure body protein and an accurate method for the determination of phenylalanine are required. In this research, the first condition was met by using carefully purified hemoglobin from both normal controls and well-documented phenylketonurics, and the second by using the techniques of ion-exchange column chromatography as developed largely by Moore and Stein (for a review see reference 11).

METHODS

Preparation of hemoglobin. Hemoglobin was prepared by the method of Drabkin (12) except for minor modifications, the most important of which was the conversion to the more stable carbonmonoxy form early in the purification.

This investigation used two preparations of hemoglobin from normal individuals and two from phenylketonuric patients. The first preparation of normal hemoglobin employed red cells from which the plasma had been removed; these cells were obtained from Hyland Laboratories, Los Angeles. The second sample of normal blood came from a known healthy donor. The possibility exists that the high levels of phenylalanine in the plasma of phenylketonuric patients may lead to adsorption of phenylalanine by the hemoglobin. In an attempt to rule out this possibility, phenylalanine was added to the whole normal blood to give a final concentration of 30 mg. per 100 ml. and the blood was allowed to stand at 20° C. for 24 hours prior to separation of the red cells. The specimens of phenylketonuric blood were provided by Drs. George Tarjan and Stanley W. Wright of the Pacific State Hospital, from patients who had been studied by them and who are described in their review (2). These patients are listed as Case Nos. 5 and 20.

Apart from the above difference, the subsequent prepa-

[1] Supported in part by a grant from the Ford Foundation.

[2] National Science Foundation Fellow 1956–1957.

ration of the four hemoglobin samples was identical. After the red cells had been separated from the plasma, they were washed twice with isotonic saline at 0° C. and CO was bubbled through the red cell suspension for 10 minutes. During the remainder of the purification all solutions were saturated with CO. The red cells were washed twice more with isotonic saline, hemolyzed with an equal volume of distilled water and 0.4 volume of toluene, and allowed to stand overnight at 1° to 2° C. The hemolyzed cells were then centrifuged at 18,000 G for 1 hour at 0° C., after which the hemoglobin layer was removed and recentrifuged in the same way. The hemoglobin solution was dialyzed against Drabkin's 2.8 M phosphate buffer initially at 37° C. and then at 1° to 2° C. After the crystals which formed overnight had been washed four times with 2.8 M phosphate buffer, they were dissolved in distilled water to give a volume equal to that of the original laked cell contents, and the crystallization was repeated. In the method of crystallization as here employed, almost no hemoglobin remains in the supernatant solution and any fractionation is thus avoided. These crystals were dissolved in distilled water and dialyzed against distilled water until the dialysate was negative for phosphates. The solution was finally dialyzed against glass distilled water, and the dialysate was negative for residue, phosphates, or ninhydrin positive material. The hemoglobin solution was then adjusted to a concentration of approximately 6 Gm. per 100 ml. Simultaneous aliquot portions were taken for determination of the concentration and for hydrolysis.

Determination of concentration. The concentration of the hemoglobin solutions was determined both by dry weight and by the spectrophotometric methods of Drabkin and Austin (13).

The determination of the dry weight was performed in quadruplicate by heating the weighed 3-ml. aliquot portions first to 90° C. for two hours (to prevent boiling and spattering) and then to 105° C. for one hour. After cooling over "Drierite" and weighing, repeated heating to 105° C. for one hour produced no further loss in weight. Values for dry weight agreed within 0.5 per cent.

The concentration of the hemoglobin was also determined spectrophotometrically as carbonmonoxyhemoglobin and as ferrihemoglobin cyanide by the methods of Drabkin (13). The concentration using these techniques was 2 to 4 per cent less than that obtained by dry weight and agreement between the two spectrophotometric determinations was no better than 1 or 2 per cent.

The dry weight was considered to be the most reliable basis for calculating the amino acid composition of the hemoglobins prepared in identical fashion. The spectrophotometric determination as carbonmonoxyhemoglobin will be less reliable if a few per cent of ferrihemoglobin is present in variable amount in the different preparations. The spectrophotometric determination as ferrihemoglobin cyanide is unsatisfactory in our work because some difficulty was experienced in converting carbonmonoxyhemoglobin to ferrihemoglobin.

Hydrolysis. Four 1-ml. aliquot portions of each hemoglobin preparation were pipetted into long-neck hydrolysis flasks containing 10 ml. of doubly distilled 6 N HCl. Two hydrolyses were refluxed for 24 hours and two for 72 hours. Each hydrolysate foamed badly for the first hour and required frequent cooling of the neck of the flask to avoid loss of material; subsequently, there was no foaming over. Capryl or n-octyl alcohol was ineffective in preventing foaming at this stage as each distilled into the condenser and remained there. At the end of the hydrolysis, the HCl was removed by vacuum distillation with a rotary evaporator at 60° C.; one drop of capryl alcohol was an effective anti-foaming agent. In order to remove traces of HCl, the residue was taken up twice with distilled water which was removed by the same technique. Finally, the residue which was not completely soluble in 0.5 M acetic acid was transferred quantitatively with this solvent to a 10-ml. volumetric flask and the solution was brought up to mark. The insoluble material which probably consisted of heme degradation products was not removed before chromatography but, rather, 1 ml. of this suspension was pipetted directly on the column.

Chromatography. In these experiments, it was essential that the phenylalanine be completely separated from all other constituents of the protein hydrolysate. Because phenylalanine is the first amino acid to emerge from starch chromatograms under certain conditions (14) this method would have been convenient, but no developer which was studied would separate phenylalanine from traces of tryptophan which survive the hydrolysis.

Chromatograms on Dowex 50 require strict temperature control to separate phenylalanine from tyrosine (15) and suffer the disadvantage that phenylalanine emerges rather late in the chromatogram. However, Hirs, Moore, and Stein (16) describe the use of a 30-cm. Dowex 1-X8 column developed with 0.5 M acetic acid for separation of tyrosine, glutamic acid, and aspartic acid and it was known (17) that on this column phenylalanine precedes tyrosine but is incompletely separated from other amino acids. Therefore, it was hoped that a longer column would achieve a complete separation of phenylalanine.

Accordingly, a 0.9 × 100-cm. column of Dowex 2-X10 [3] was poured and found to separate phenylalanine from the remaining amino acids adequately for most purposes but not quite enough for these experiments. When the length of the column was increased to 200 cm., the phenylalanine separated completely from the remaining amino acids. The identity and purity of the zone of phenylalanine were proved by chromatographing a hemoglobin hydrolysate on the 200-cm. column. After those fractions which contained the phenylalanine were combined, the compound was dinitrophenylated and the DNP-derivative was chromatographed on silicic acid-Celite by the method of Green and Kay (19). The DNP-derivative was found to be pure DNP-phenylalanine in amounts equivalent to the

[3] The behavior of amino acids on Dowex 2-X10 is almost the same as that on Dowex 1-X8 and the former resin has been used for tyrosine determinations (18).

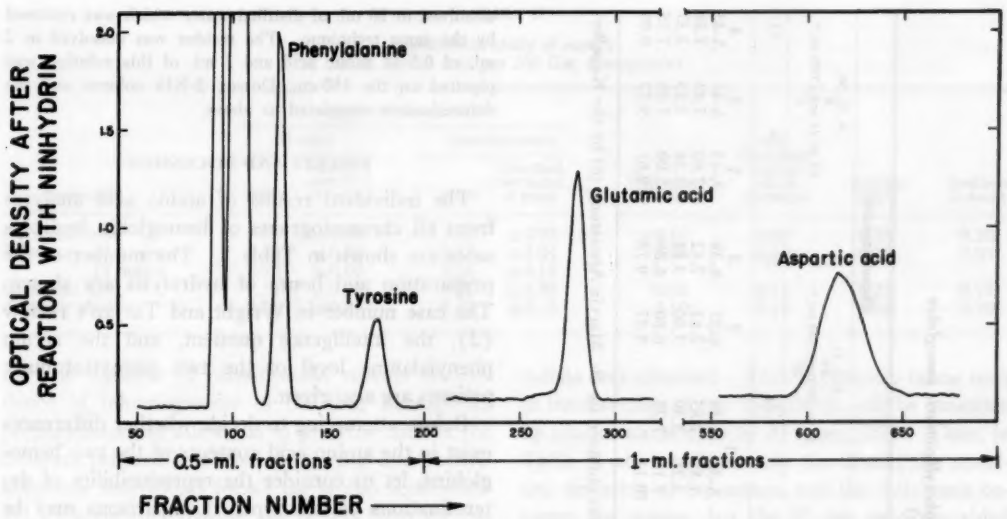


FIG. 1. SEPARATION OF SOME AMINO ACIDS IN A COMPLETE HYDROLYSATE OF ADULT HUMAN HEMOGLOBIN BY CHROMATOGRAPHY ON DOWEX 2-X10 WITH 0.5 M ACETIC ACID AS DEVELOPER

phenylalanine determined by a duplicate chromatogram on the Dowex 2-X10 column.

The Dowex 2-X10 which was used for these columns had been purified essentially in the manner described by Hirs, Moore, and Stein (16) for Dowex 1-X8 and had been passed wet through a 200-mesh sieve.

As mentioned above, a 1-ml. portion of hydrolysate was placed on the column. After this sample had penetrated into the column, it was rinsed in with 3×0.5 ml. of 0.5 M acetic acid. Development was carried out with 0.5 M acetic acid. At the beginning of the chromatogram, the flow rate of developer was set at 3.5 ml. per hour by adjusting the position of the solvent reservoir in order to apply the necessary hydrostatic pressure. After 200 0.5-ml. fractions had been collected (well past the tyrosine peak) 18 cm. of mercury pressure was applied to increase the flow rate to 7 ml. per hour and 120 1-ml. fractions were collected until emergence of the peak of glutamic acid was complete. The next 230 ml. of effluent were not collected in fractions because of the absence of zones and finally 120 more 1-ml. fractions were taken to collect the aspartic acid. After the completion of the chromatogram, the upper 2 cm. of the column, which contained the brown products of the hydrolysis, were removed and replaced with fresh resin. Then, 100 to 200 ml. of 0.5 M acetic acid were passed through the column before its next use.

The original ninhydrin method of Moore and Stein (20) was used to determine the amino acid content of the effluent fractions after they had been brought to pH 5 with N NaOH (0.1 ml. or 0.2 ml., depending on the size of the fraction). The color yields of phenylalanine, tyrosine, glutamic acid, and aspartic acid were redetermined and found to be 0.92, 0.93, 1.04, and 0.97, respectively. Four chromatograms with known phenylalanine in amounts of the same magnitude as was present in the hydrolysates yielded quantitative recovery with ± 3 per cent precision. The tyrosine and glutamic acid were also recovered quantitatively, but only about 85 per cent of the aspartic acid was recovered from known amounts of amino acid, or when added to a hydrolysate in which the aspartic acid had previously been determined.

A typical chromatogram is illustrated in Figure 1. The optical density of the individual fractions after reaction with ninhydrin is plotted against the fraction number. The fractions with optical density above 1.00 were read at an appropriate dilution, but, before plotting, the optical density was calculated to what would have been observed in the same volume as the blank fractions. The peak of the phenylalanine appears at 60 ml. of effluent volume, tyrosine at 83 ml., glutamic acid at 176 ml., and aspartic acid at 517 ml. Tryptophan is found in 24-hour hydrolysates as a small zone with its peak at 151 ml. As it produces only a slight increase of optical density readings above the blank an estimate of the amount of tryptophan could rarely be obtained, although generally there was evidence for its presence as in the figure.

Determination of serum phenylalanine. The phenylalanine content in the phenylketonuric serum was determined in the following way: A 2-ml. sample of the patient's serum was diluted to 10 ml. with distilled water and the proteins were precipitated with 10 ml. of 10 per cent trichloracetic acid. The precipitate was washed twice with 5 ml. of 5 per cent trichloracetic acid, and the combined supernatant solution and washings were extracted five times with an equal volume of ether to remove the trichloracetic acid. The aqueous layers were then concentrated to dryness at 50° C. in a rotary evaporator and

TABLE I

Individual values for the contents of several amino acids in normal and phenylketonuric hemoglobin (Gm. of amino acid per 100 Gm. of hemoglobin)

Type	Normal										Phenylketonuric									
Individual	1. Bank blood					2. M. C.					3. J. G.					4. G. M.				
I.Q.											49					8				
Serum phenylalanine											29 or 39 mg./100 ml.*					13 or 21 mg./100 ml.*				
Case number											20					5				
Hours hydrolysis	24				72		24		72		24			72		24		72		
Hydrolysate no.	1		2		3		1	3	4		1									
Chromatogram no.	A-27	A-30	A-28	A-31	A-29	A-32	A-33	A-34	A-35		A-36	A-39		A-37	A-38	A-43	A-44	A-45		
Phenylalanine	7.56	7.39	7.75	7.43	7.39	7.51	7.69	7.46	7.46		7.53	7.85		7.42	7.42	7.62	7.44	7.58		
Tyrosine	3.08	3.50	3.37	3.39	3.15	3.14	3.15	3.22	3.00		3.21	3.39		3.30	3.11	3.24	3.13	3.37		
Glutamic acid	7.06	7.06	7.37	7.00	7.09	7.18	7.03	7.06	7.10		7.16	7.27		6.80	6.88	7.09	7.16	7.25		
Aspartic acid	9.82	9.56	9.83	9.70	9.36	9.79	9.41	9.99	9.57		10.11			9.37	9.78	9.61	9.77	9.99		

* The lower value was obtained by the procedure described in Methods, whereas the higher value was obtained by Dr. Marvin Armstrong (6) by the Kapeller-Adler method.

dissolved in 10 ml. of distilled water which was removed by the same technique. The residue was dissolved in 2 ml. of 0.5 M acetic acid and 1 ml. of this solution was pipetted on the 100-cm. Dowex 2-X10 column and the determination completed as above.

RESULTS AND DISCUSSION

The individual results of amino acid analyses from all chromatograms of hemoglobin hydrolysates are shown in Table I. The number of the preparation and hours of hydrolysis are shown. The case number in Wright and Tarjan's review (2), the intelligence quotient, and the serum phenylalanine level of the two phenylketonuric patients are also given.

Before attempting to decide whether differences exist in the amino acid contents of the two hemoglobins, let us consider the reproducibility of determinations of this type. Comparisons may be made of the results from duplicate determinations on the same hydrolysate, from hydrolysates of the same preparation of hemoglobin, and from different preparations of hemoglobin. Thus, we may compare, respectively, the results of Chromatograms No. A-27 and A-30 (Table I), of these and Chromatograms No. A-28 or A-31, and finally, of all the foregoing with Chromatogram No. A-33. It is apparent that duplicate determinations on the same hydrolysate agree no better than duplicate determinations in which still other variables are present. These three variables, then, have no specific influence on the results. The individual determinations of phenylalanine and glutamic acid agree within the range of ± 3 per cent usual in column chromatography. The error for tyrosine exceeds this. Part of the error in the tyrosine determinations may be due to the smaller quantities of this amino acid, which make any error in the blank more significant. The variation in the values for aspartic acid is large and is quite likely related to its destruction on the column.

In Table II are shown the results of a statistical study of the data [4] (21). An analysis of variance

[4] In Chromatogram No. A-39, a further attempt to assess the extent of the destruction of aspartic acid was made. For this experiment, a portion of the same hydrolysate that was used for Chromatogram No. A-36 was placed on the column and followed immediately by a known amount of aspartic acid solution. The resulting chromatogram was unusual in that the optical density was abnormally high between phenylalanine and the pre-

TABLE II

Statistical study of results
(Gm. amino acid per 100 Gm. hemoglobin)

Amino acid	Type of hemoglobin: Normal Mean	Normal Standard deviation of mean	Phenylketonuric Mean	Phenylketonuric Standard deviation of mean	Difference in means	95% Confidence limit of diff. in the means	Residue weight	Residues in doubt
Phenylalanine	7.52	±0.05	7.50	±0.03	−0.02	0.09	0.25	0.36
Tyrosine 24 hours	3.30	±0.08	3.22	±0.04	0.08	0.25	0.27	0.93
72 hours	3.13	±0.05	3.24	±0.13				
Glutamic acid	7.11	±0.04	7.06	±0.06	0.05	0.17	0.22	0.77
Aspartic acid	9.67	±0.07	9.77	±0.12	−0.10	0.14	0.20	0.70

for one variable of classification reveals no evidence of inhomogeneity of the four hemoglobin preparations and allows us to pool the data of the different preparations from the normal and phenylketonuric individuals for all the amino acids studied. Except for tyrosine, the results from the 24-hour and 72-hour hydrolysates do not differ. A "t" test on the combined data from both types of hemoglobin reveals that there is less than a 20 per cent probability that the smaller value of tyrosine in the 72-hour hydrolysates is due to chance alone. Hence, although the data do not reveal a significant difference by usual standards, it is unjustifiable in the case of this amino acid to pool the results on 24-hour and 72-hour hydrolysates in the light of other evidence that tyrosine is lost on prolonged hydrolysis (22).

In order to test the hypothesis that phenylketonuric hemoglobin has an increased amount of phenylalanine, and, as a corollary, a diminished amount of other amino acids, we take the difference between the means, subtracting the mean value of phenylalanine content for normal hemoglobin from that of phenylketonuric hemoglobin, and, in the case of the other amino acids, subtracting the phenylketonuric amino acid content from the value for normal hemoglobin (see column 5, Table II). The differences are small, and, taking into account the variation of the individual measurements, the hypothesis would probably be rejected without any statistical treatment. However, to evaluate more closely the effect of variation of individual measurements, the 95 per cent one-sided confidence limit of the difference in means was obtained. This difference, taken only in the direction of our hypothesis, will be exceeded by chance alone once in 20 times (21). Thus, in Table II are found not only the means, the standard deviation of the means, and the difference between the means, but the 95 per cent one-sided confidence limit of the difference. For comparison, in the next column the weight of each amino acid in Gm. per 100 Gm. of hemoglobin equivalent to one residue per molecule is given, and finally, in the last column, the quotient of the confidence limit of the difference by the residue weight is given. This quotient provides an estimate of the number of residues of amino acid in doubt. There is less than one chance in 20 that phenylketonuric hemoglobin exceeds normal hemoglobin by as much as 0.36 residue. At the 99.5 per cent level of significance there is less than one chance in 200 that phenylketonuric hemoglobin exceeds normal hemoglobin by as much as 0.72 residue. It is also evident that there is less than one chance in 20 that phenylketonuric hemoglobin has one residue less of tyrosine, glutamic acid or aspartic acid.

These results must not be interpreted to mean that the absolute amount of phenylalanine in hemoglobin is known with such accuracy because it does not take into account the possibility of constant errors such as errors in determination of the concentration of hemoglobin, the color yields by the ninhydrin procedure, and so forth. However, because two materials prepared and analyzed in the same way are compared, such constant errors are cancelled and this precision is possible in the comparison.

A comparison of the amino acid content of normal hemoglobin with the results of other workers

ceding peak. Because of the unusual procedure and result, the determinations of this chromatogram have been omitted from the statistical data of Table II.

TABLE III

Comparison of results with those of previous workers (Gm. of amino acid per 100 Gm. of hemoglobin)

	This paper	Schroeder, Kay and Wells (23)	Van der Schaaf and Huisman (22)	Rossi-Fanelli *et al.* (24)
Phenylalanine	7.52	7.66	7.93	9.62
Tyrosine 24 hours	3.30	2.99	4.40	2.90
72 hours	3.13			
Glutamic acid	7.11	7.17	7.20	7.41
Aspartic acid	11.40*	11.02	10.60	9.99

* Corrected for 15 per cent loss on the column.

is seen in Table III (22–24). Aside from the divergent reports of Van der Schaaf and Huisman (22) for tyrosine, and of Rossi-Fanelli, Cavallini, and DeMarco (24) for phenylalanine and aspartic acid, the results are in good agreement. Schroeder, Kay, and Wells (23) report a slightly higher value for phenylalanine than was found in the present study, but their phenylalanine was contaminated with tryptophan which was not removed by starch chromatography. If the amount of tryptophan that survived acid hydrolysis and is present in Chromatogram No. A-33 is calculated as phenylalanine, its quantity is 0.13 Gm. per 100 Gm. of hemoglobin. If this amount is subtracted from Schroeder, Kay, and Wells' value for phenylalanine content of 7.66, the result, 7.53, is in exceptional agreement with the present value of 7.52.

The aspartic acid has been corrected for the loss that is known to take place on the column, but the result reported here is less reliable than that of other workers.

CONCLUSIONS

It may be concluded from the results of the present work that, if normal and phenylketonuric hemoglobin are of uniform phenylalanine content, they do not differ by as much as one residue of phenylalanine per molecule. It is possible that some of the molecules of the phenylketonuric hemoglobin have one or more extra residues of phenylalanine but the accuracy of the present technique is unable to detect a difference in the average phenylalanine content of much less than one residue per molecule.

Further work is being carried out in these laboratories on the phenylalanine content of other body proteins. If such investigations yield results similar to the above, it would render unlikely the possibility that phenylalanine acts as a toxic substance by accumulating to an abnormally large extent in proteins. This indicates that either the cells can exclude from sites of protein synthesis the high levels of phenylalanine found in body fluids, or that the gene-controlled protein synthesis is remarkably efficient.

SUMMARY

1. One possible mechanism for phenylalanine toxicity in phenylketonuria is the abnormal accumulation of phenylalanine in body proteins and the displacement of other amino acids. A vital protein containing a single extra residue of phenylalanine might function abnormally and produce the clinical symptomatology.

2. The phenylalanine, tyrosine, glutamic acid and aspartic acid content of two samples of hemoglobin from normal individuals and two samples from phenylketonuric patients have been compared. The amino acid determinations were made chromatographically on a 200-cm. column of Dowex 2-X10, an anion exchange resin, with 0.5 M acetic acid as a developer.

3. The results show no significant difference between normal and phenylketonuric hemoglobin. Moreover, there is less than one chance in 200 that the phenylalanine content of phenylketonuric hemoglobin exceeds that of normal hemoglobin by as much as one residue of phenylalanine, and less than one chance in 20 that phenylketonuric hemoglobin has one residue less of tyrosine, glutamic acid, or aspartic acid.

4. From the experimental evidence, the phenylalanine toxicity in phenylketonuria is not associated with any abnormal accumulation of phenylalanine in hemoglobin.

ACKNOWLEDGMENTS

The authors wish to thank Professor Linus Pauling for suggesting the topic of this research, Drs. George Tarjan and Stanley W. Wright for their assistance in obtaining specimens of phenylketonuric blood, and Dr. Marvin D. Armstrong for his determinations of the serum phenylalanine level of the patients studied.

REFERENCES

1. Jervis, G. A., Phenylpyruvic oligophrenia (phenylketonuria). Genetics and the inheritance of integrated neurological and psychiatric patterns.

Proc. A. Research Nerv. & Ment. Dis., 1954, **33**, 259.

2. Wright, S. W., and Tarjan, G., Phenylketonuria. Am. J. Dis Child., 1957, **93**, 405.
3. Jervis, G. A., Phenylpyruvica oligophrenia deficiency of phenylalanine-oxidizing system. Proc. Soc. Exper. Biol. & Med., 1953, **82**, 514.
4. Udenfriend, S., and Bessman, S. P., The hydroxylation of phenylalanine and antipyrine in phenylpyruvic oligophrenia. J. Biol. Chem., 1953, **203**, 961.
5. Bickel, H., Gerrard, J., and Hickmans, E. M., Influence of phenylalanine intake on chemistry and behaviour of a phenylketonuric child. Acta paediat., 1954, **43**, 64.
6. Armstrong, M. D., and Tyler, F. H., Studies on phenylketonuria. I. Restricted phenylalanine intake in phenylketonuria. J. Clin. Invest., 1955, **34**, 565.
7. Pauling, L., The probability of errors in the process of synthesis of protein molecules *in* Festschrift Prof. Dr. Arthur Stoll. Basel, Birkhäuser, 1957, p. 597.
8. Block, R. J., Jervis, G. A., Bolling, D., and Webb, M., Chemical and metabolic studies on phenylalanine. III. The amino acid content of tissue proteins of normal and phenylpyruvic oligophrenic individuals. A note on the estimation of phenylalanine. J. Biol. Chem., 1940, **134**, 567.
9. Schrappe, O., Zur pathologischen Physiologie des Phenylbrenztraubensäure-Schwachsinns. Nervenarzt, 1952, **23**, 175.
10. Keup, W., Die Amino säure-Bausteine körpereigener Proteine bei Oligophrenia phenylpyruvica. Monatsschr. f. Psychiat. u. Neurol., 1955, **130**, 299.
11. Schroeder, W. A., Column chromatography in the study of the structure of peptides and proteins. Progr. Chem. Org. Nat. Prod., 1954, **11**, 240.
12. Drabkin, D. L., A simplified technique for a large scale crystallization of human oxyhemoglobin. Isomorphous transformations of hemoglobin and myoglobin in the crystalline state. Arch. Biochem., 1949, **21**, 224.
13. Drabkin, D. L., and Austin, J. H., Spectrophotometric studies. I. Spectrophotometric constants for common hemoglobin derivatives in human, dog, and rabbit blood. J. Biol. Chem., 1932, **98**, 719.
14. Stein, W. H., and Moore, S., Chromatography of amino acids on starch columns. Separation of phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine. J. Biol. Chem., 1948, **176**, 337.
15. Moore, S., and Stein, W. H., Chromatography of amino acids on sulfonated polystyrene resins. J. Biol. Chem., 1951, **192**, 663.
16. Hirs, C. H. W., Moore, S., and Stein, W. H., The chromatography of amino acids on ion exchange resins. Use of volatile acids in elution. J. Am. Chem. Soc., 1954, **76**, 6063.
17. Hirs, C. H. W., Personal communication.
18. Schroeder, W. A., and Kay, L. M., The amino acid composition of *Bombyx mori* silk fibroin and of Tussah silk fibroin. J. Am. Chem. Soc., 1955, **77**, 3908.
19. Green, F. C., and Kay, L. M., Separation of sixteen dinitrophenylamino acids by adsorption chromatography on silicic acid-Celite. Anal. Chem., 1952, **24**, 726.
20. Moore, S., and Stein, W. H., Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem., 1948, **176**, 367.
21. Dixon, W. J., and Massey, F. J., Jr., Introduction to Statistical Analysis, Second Edition. New York, McGraw-Hill Book Company, Inc., 1957.
22. Van der Schaaf, P. C., and Huisman, T. H. J., The amino-acid composition of human adult and foetal carbonmonoxyhaemoglobin estimated by ion exchange chromatography. Biochim. et biophys. acta, 1955, **17**, 81.
23. Schroeder, W. A., Kay, L. M., and Wells, I. C., Amino acid composition of hemoglobins of normal negroes and sickle-cell anemics. J. Biol. Chem., 1950, **187**, 221.
24. Rossi-Fanelli, A., Cavallini, D., and DeMarco, C., Amino-acid composition of human crystallized myoglobin and haemoglobin. Biochim. et biophys. acta, 1955, **17**, 377.

PHYSIOLOGICAL DISPOSITION OF 4-C^{14}-CORTISOL DURING LATE PREGNANCY[1]

By CLAUDE J. MIGEON, JEAN BERTRAND,[2] AND PATRICIA E. WALL

(From the Department of Pediatrics, The Johns Hopkins Hospital, Baltimore, Md.)

(Submitted for publication January 18, 1957; accepted May 16, 1957)

The plasma levels of free 17-hydroxycorticosteroids (17-OH-CS) were found to rise progressively during pregnancy (1–5). In cases of vaginal delivery and "indicated cesarean section," labor and delivery had a tendency to further increase the maternal concentrations (6, 7), while in cases of "elective, repeat cesarean section," the levels were not as high as those observed in cases where labor had taken place (8, 7).

The levels of 17-OH-CS in cord blood were observed to be very low in cases of "elective, repeat cesarean section" (8, 7). In cases of vaginal delivery and indicated cesarean section the concentrations were as high or higher than those of normal adults (3, 6–9).

Studies involving the intravenous infusion of ACTH or cortisol to patients selected for "elective, repeat cesarean section" strongly suggested that 17-OH-CS could cross the "placental barrier" from the mother to the baby (7). In all cases, there was a direct relationship between maternal and cord levels, the latter values being one-half to one-fifth of those of the corresponding mother (6, 7).

These findings suggested that, near term, the fetal adrenals produce little 17-OH-CS and that the increase in cord levels observed in cases of either vaginal delivery or "indicated cesarean section" was the result of elevated maternal concentrations.

The present work is an attempt to elucidate further the metabolism of corticosteroids during pregnancy. It was thought that radioactive cortisol might be useful since it would permit a dynamic study without interfering with the endogeneous production of steroid.

METHODS

Preliminary study on newborn infants. If, following the injection of radioactive material to a mother, a large part of the dose were to cross the placenta, we had to be assured that the newborn would dispose of the compound as rapidly as do normal adults (10–13). Therefore, in a first step of the present study, we measured the urinary excretion of radioactivity during the 48-hour period following the administration of a minimal dose of 4-C^{14}-cortisol (1.5×10^{-3} microcurie) to two newborns, 15 and 20 hours of age. Since, as shown in the Results section, the 48-hour excretion of radioactivity was within normal limits, it was considered safe to proceed with the injection of mothers shortly before delivery.

Experimental subjects. The other subjects of the study were nine pregnant females, 20 to 38 years of age. An "elective, repeat cesarean section" was performed approximately at term and before onset of labor in all cases. Sections were deemed advisable because of a previous "indicated section," but otherwise the pregnancies had been normal. The surgery was performed with local infiltration of Novocain® and intravenous Pentothal® anesthesia.

Purity of 4-C^{14}-cortisol. The 4-C^{14}-cortisol was provided by the Endocrinology Study Section, Division of Research Grants, National Institutes of Health, Bethesda, Maryland. Its specific activity was 1.467 millicuries per millimole. The purity of the compound was tested as previously described (13), and 90 to 95 per cent of the radioactivity was found to move as cortisol on our paper chromatograms when using the Bush (14) system of benzene : methanol : water (100 : 55 : 45).

Mode of administration of 4-C^{14}-cortisol. The radioactive steroid was dissolved in re-distilled ethanol so that 1 ml. of the solution contained approximately 1 microcurie. For human administration, 1 ml. of this solution was carefully pipetted out and diluted with 10 ml. of 5 per cent dextrose in water. The method of intravenous injection and the mode of calculation of the dose actually administered were similar to those previously described (13). The dose given to each of the subjects of the present study is indicated in Table I.

Subject A. O. received the injection 24 hours, and subjects A. P. and A. Q., 48 and 46 hours, respectively, prior to delivery. In the six other cases the steroid was given from 17 to 69 minutes before delivery (see Table I).

[1] This work was made possible by a Grant-in-Aid from the American Cancer Society of the Committee on Growth of the National Research Council, and by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

[2] Recipient of a research fellowship of the French government and of a Fulbright Travel Fellowship.

TABLE I

Radioactivity readily extractable from plasma with chloroform, and that extractable following β-glucuronidase hydrolysis at various intervals following 4-C^{14}-cortisol administration (expressed as percentage of the administered dose per liter of plasma)

SUBJECTS		TIME AFTER INJECTION	% DOSE PER LITER PLASMA	
			FREE FRACTION	GLUCUROMOSIDE FRACTION
A.P.	MOTHER, 21 yrs., 82 kg. 779,600 c/m, 48 hrs. prior to delivery	30'	5.04	0.36
		60'	4.09	0.29
		120'	3.26	0.46
		240'	1.85	0.46
		48 hrs.	0.	0.
	BABY ♀, 3670 g.	48 hrs.	0.	0.
A.Q.	MOTHER, 24 yrs., 69 kg. 812,000 c/m, 46 hrs. prior to delivery	20'	6.20	0.40
		60'	4.51	0.44
		120'	3.05	0.39
		240'	1.75	0.43
		46 hrs.	0.	0.
	BABY ♀, 3050 g.	46 hrs.	0.	0.
A.O.	MOTHER, 26 yrs., 65 kg. 766,500 c/m, 24 hrs. prior to delivery	30'	4.75	0.35
		60'	3.55	0.56
		140'	2.47	0.48
		250'	1.45	0.54
		24 hrs.	0.	0.
	BABY ♂, 2810 g.	24 hrs.	0.	0.
A.G.	MOTHER, 23 yrs., 65 kg. 904,700 c/m, 17' prior to delivery	17'	7.70	0.46
		27'	5.86	0.33
		150'	---	0.31
		260'	1.85	0.52
	BABY ♂, 2690 g.	17'	1.61	0.13
A.H.	MOTHER, 36 yrs., 70 kg. 872,500 c/m, 27' prior to delivery	27'	4.19	0.34
		60'	3.75	0.32
		145'	3.04	0.30
		263'	1.63	0.38
	BABY ♀, 2680 g.	27'	1.46	0.09
A.E.	MOTHER, 38 yrs., 76 kg. 907,100 c/m, 39' prior to delivery	20'	5.14	0.47
		39'	3.46	0.19
		150'	1.49	0.19
		240'	1.15	0.16
	BABY ♀, 2800 g.	39'	1.21	0.09
A.L.	MOTHER, 22 yrs., 76 kg. 936,500 c/m, 51' prior to delivery	17'	6.42	0.30
		37'	4.62	0.31
		51'	3.29	0.38
		120'	2.50	0.35
		240'	1.70	0.37
	BABY ♂, 3200 g.	51'	1.57	0.08
A.I.	MOTHER, 25 yrs., 93 kg. 814,200 c/m, 60' prior to delivery	15'	6.26	0.48
		60'	4.11	0.48
		120'	2.27	0.43
		240'	1.17	0.36
	BABY ♂, 2090 g.	60'	0.80	0.13
A.M.	MOTHER, 24 yrs., 50 kg. 900,000 c/m, 69' prior to delivery	20'	6.80	0.33
		69'	4.64	0.56
		120'	3.98	0.56
		240'	2.46	0.63
	BABY ♂, 3200 g.	69'	1.70	0.24

Collection of blood and urine samples. Samples of maternal blood were drawn in heparinized syringes at various times after the injection. In all cases, a sample was collected at the exact time of delivery in addition to a specimen of cord blood. The plasma was immediately separated from the red blood cells. Urine samples were

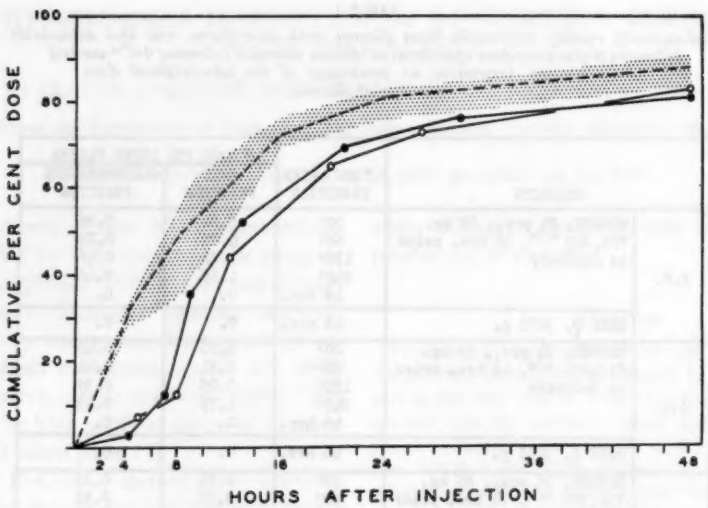


FIG. 1. CUMULATIVE URINARY EXCRETION OF RADIOACTIVITY FOLLOWING 4-C^{14}-CORTISOL INJECTION IN TWO NORMAL NEWBORNS, 15 AND 20 HOURS OF AGE

The broken line is the average urinary excretion of seven normal young adults (13, 15). The shaded area shows the range of values for these normal individuals. The full lines join the values obtained in the two normal newborns.

collected from the mothers and their babies at specified times following cortisol administration (see Table I).

Preparation and assay of the various plasma and urine extracts. Each plasma sample was extracted three times with freshly re-distilled chloroform (40 ml. × 3) in order to extract the free steroids ("free fraction"), after which 100 ml. of absolute ethanol was added to the plasma residue. After shaking and centrifuging, the liquid phase was decanted and the remainder was washed with 50 ml. of ethanol. The pooled ethanol extracts were then evaporated to dryness. The ethanol residue was dissolved in 20 ml. of distilled water, incubated with β-glucuronidase (approximately 500 units per ml. of plasma), at pH 4.6 (acetate buffer), at 37° C. for 48 hours.[3] Extraction was then performed with chloroform (40 ml. × 3) to obtain the steroids formerly present as conjugates of glucuronic acid ("glucuronoside fraction").

A 25-ml. aliquot of each urine was extracted three times with an equal volume of chloroform in order to obtain the "free fraction." A β-glucuronidase hydrolysis was then carried out on the urine residue under the same conditions regarding temperature and pH as for the plasma ("glucuronoside fraction"), followed by 48-hour continuous extraction with ether at pH 0.8. Three ml. of concentrated hydrochloric acid was then added to the aqueous residue, the mixture boiled for 20 minutes and then extracted three times with its volume of chloroform.

A great discrepancy between the total urinary radioactivity and the sum of those found in the various urinary fractions was observed in all cases, and for this reason a count of the isotopic activity was made on the urine residues following the removal of "free," "glucuronoside" and "continuous ether extraction" fractions of patients A. M. and A. O. The urine residues of patient A. G. were extracted twice with equal volumes of butanol and the butanol extracts were assayed for radioactivity.

All extracts of plasma and urine, as well as whole urine were assayed for radioactivity in the manner previously described (13).

Some of the "free fractions" prepared from maternal and cord plasma were chromatographed on paper using the Bush (14) system, benzene: methanol: water (100: 55: 45). The following areas of the chromatogram were cut out and eluted with ethanol: 1) the origin, 2) the zone between the origin and the cortisol area, 3) the cortisol area, 4) the zone between the cortisol area and that of dihydrocortisone, the latter area being included in this zone, 5) the rest of the chromatogram with the front included. The ethanol extract was then assayed for radioactivity content.

Control subjects for the present study. The results obtained in a group of five normal young adult males of a previous study (13) and in two normal non-pregnant females (15) were used in the present work as control values. The two normal non-pregnant females (subjects A. R. and B. A.) were 28 and 31 years of age. The radioactive steroid was administered to subject A. R. 19 days and to subject B. A. 15 days after the beginning of the last period. Plasma and urine values of subject B. A. were identical to the various mean values for the group

[3] Ketodase, a beef liver preparation, from Warner-Chilcott Laboratories was used in the present study.

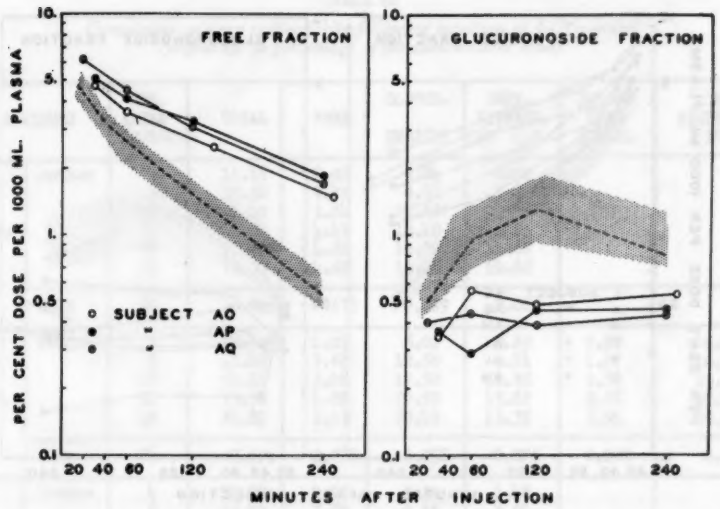


FIG. 2. PLASMA RADIOACTIVITY IN THE "FREE" AND "GLUCURONOSIDE" FRACTIONS OF MOTHERS WHO RECEIVED 4-C^{14}-CORTISOL 24 TO 48 HOURS PRIOR TO AN ELECTIVE, REPEAT CESAREAN SECTION

The broken line joins the average of the values obtained in five normal young adult males (13) and two normal young adult non-pregnant females (15). The shaded area shows the range of values for these normal individuals. The full lines join the values obtained in subjects A. O., A. P. and A. Q.

of five normal males. Some of the urinary levels of subject A. R. were slightly different from the corresponding mean of the normal males, but they were all in the range of variation of the male group. Furthermore, sex did not appear to influence the removal of non-isotopic cortisol in a control group of 26 young adults, comprising 12 non-pregnant females and 14 males. Consequently, it was thought possible to compare the 4-C^{14}-cortisol studies in pregnant women with those carried out in the two non-pregnant females as well as in the male group.

Our results, for control subjects, are similar to those reported by other authors (11). When Peterson and Wyngaarden (12) studied the specific activity of cortisol in plasma, instead of the total free radioactivity, they obtained a wider range of variance for the values of half-life. This might be due to the fact that the error of the chemical technique adds itself to that of the isotopic assay, while the latter one is only involved in the study of total free plasma radioactivity (11, 13).

RESULTS

Rate of elimination of radioactivity by newborn infants

The cumulative urinary excretion of radioactivity following 4-C^{14}-cortisol infusion to two newborns is shown in Figure 1 and is compared to the average excretion of five normal males (13) and two normal females (15). It can be seen that, after an initial lag period of 4 to 8 hours, the rate of elimination of radioactivity by the two babies was similar to that observed in adults, and 48 hours after the injection the cumulative excretions were 81.2 and 83 per cent of the dose.

Radioactivity in the "free" and "glucuronoside" fractions from maternal and cord plasma

The amount of radioactivity readily extractable from maternal plasma by chloroform following 4-C^{14}-cortisol administration is shown in Table I. The results are expressed in per cent of the administered dose per liter of plasma. These values were plotted in Figures 2 and 3, and were compared to the average curve obtained from seven normal young adults (13, 15). In most cases the logarithms of the values between the first and fourth hours fell approximately on a straight line; during this period of time, equilibrium probably existed between the various compartments of the free steroid pool of the body. The average half-

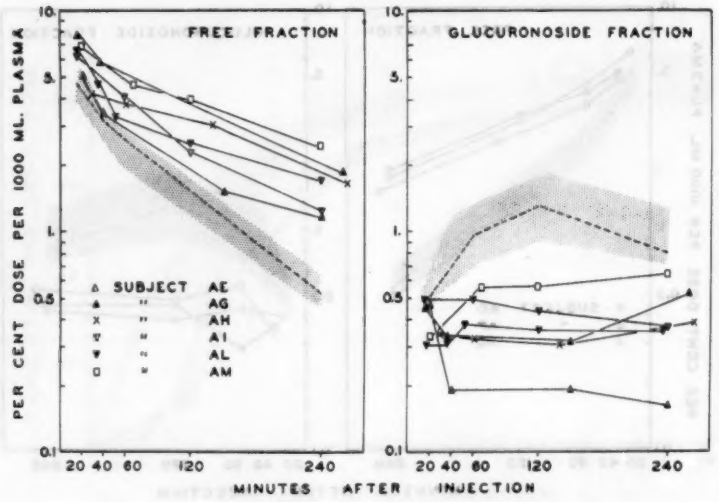


FIG. 3. PLASMA RADIOACTIVITY IN THE "FREE" AND THE "GLUCURONOSIDE" FRACTIONS OF MOTHERS WHO RECEIVED 4-C^{14}-CORTISOL 17 TO 69 MINUTES PRIOR TO ELECTIVE, REPEAT CESAREAN SECTION

life of the free fraction was then 140 minutes with a range of 100 to 180 minutes, compared to 80 minutes for the control group.

Six hours after the injection of 4-C^{14}-cortisol, the free fraction was still 1.25 per cent of the dose per liter of plasma in patient A. Q., while it was only 0.34 per cent (subject B. A.) and 0.20 per cent (normal male) in two control individuals.

Table I and Figures 2 and 3 also show the radioactivity extractable from plasma after β-glucuronidase hydrolysis. Two hours after the injection this fraction was approximately one-half or less of that found in normal subjects.

The simultaneous values for maternal and cord plasma are shown in Table I. Their ratio was from 2.09 (subject A. L.) to 5.14 (subject A. I.)

TABLE II

Paper chromatographic study of the radioactivity readily extractable from plasma with chloroform

Plasma samples			Radioactivity in per cent of total c.p.m. applied on chromatogram					
			Chromatogram area					
Subjects		Minutes after injection	No. 1 (Origin)	No. 2	No. 3 (Cortisol)	No. 4	No. 5 (Front)	Per cent recovery
B. A.	Control—Non-pregnant young adult female	20	1.8	16.4	48.2	24.0	0.2	89.6
		60	2.5	18.3	45.0	30.2	0	96.0
		120	3.3	21.0	46.7			
		240	2.4	17.6	50.2	28.1	0	98.3
A. E., A. G., A. I. (Pool)*	Maternal	15–20	2.8	12.6	53.5	24.5	0	93.4
		20–60 (Deliv.)	2.0	20.5	57.0	35.0	0	114.5
		120–240	4.3	27.0	51.0	20.4	0.1	102.8
	Cord	20–60	0	16.0	42.5	30.6	0	89.1
A. O., A. P. (Pool)†	Maternal	30	4.8	22.0	50.7	18.0	0	95.5
		60	3.2	24.4	46.9	24.1	0	98.6
		120–140	2.9	20.2	50.5	19.3	0	92.9
		240	4.6	26.0	55.1	16.6	0	102.3

* 4-C^{14}-cortisol administered 20 to 60 minutes prior to delivery.
† 4-C^{14}-cortisol administered 24 and 48 hours prior to delivery.

TABLE III

Cumulative urinary radioactivity following injection of 4-C^{14}-cortisol (expressed as percentage of the administered dose)

PATIENTS		HRS. AFTER INJECT.	TOTAL	FREE	GLUCURONOSIDE	CONT. EXTRACT. pH 0.8	STRONG ACID HYDROL.	UNACCOUNTED FOR
A.P.	Mother	4	11.15	0.96	3.04	2.82		
		8	36.50	2.77	9.82	7.85		
		12	55.40	3.64	14.60	10.42		
		24	68.00	4.49	18.10	13.85		
		48	71.90	4.62	19.09	14.62		
		72	72.30	4.62	19.25	14.62		
	Baby	24	0.045	0.(?)	0.009	0.013		
A.O.	Mother	4	16.80	1.09	5.02	3.69	0.92	6.08
		8	43.40	2.68	12.50	9.51	1.97	16.74
		12	59.50	3.08	17.50	12.85	2.70	23.37
		24	73.50	4.05	22.50	15.82	3.65	27.48
		48	74.80	4.19	23.40	16.35	3.95	26.91
	Baby	24	0.406	0.012	0.058	0.076	0.022	0.238
A.O.	Mother	4	11.50	0.90	3.39	1.48		
		8	36.40	5.60	9.75	6.64		
		12	48.3	6.21	13.82	8.71		
		24	61.2	6.70	17.50	11.00		
		48	64.4	6.75	18.22	11.24		
	Baby	24	1.037	0.035	0.102	0.150		
		48	1.626	0.044	0.141	0.217		
A.H.	Mother	4	11.38	0.83	3.60	2.30	0.58	4.07
		8	32.60	2.12	9.67	7.48	1.48	11.85
		12	42.60	2.65	12.50	9.28	3.85	14.32
		24	52.80	2.99	15.60	11.45	4.95	17.81
		48	57.0	3.14	17.7	12.55	5.07	18.54
	Baby	24	—	—	—	—	—	—
		48	0.409	0.014	0.025	0.049	0.021	0.300
A.E.	Mother	4	12.60	0.87	3.39	1.69	0.70	5.95
		8	33.90	3.39	7.84	5.71	1.34	15.62
		12	59.20	4.94	13.45	7.75	3.78	29.28
		24	75.20	5.85	16.45	9.48	5.21	38.21
		48	78.80	5.98	17.0	9.87	5.21	40.74
	Baby	24	1.220	0.024	0.048	0.096	0.096	0.956
A.M.	Mother	4	12.55	1.34	3.00	2.16		
		8	36.8	2.94	9.09	8.28		
		12	57.2	4.25	14.82	11.20		
		24	68.2	4.55	18.30	13.90		
		48	71.6	4.58	19.20	14.80		
	Baby	24	2.022	0.049	0.296	0.390		
		48	2.435	0.053	0.330	0.461		

for the free fraction, and from 2.11 (subject A. E.) to 4.75 (subject A. L.) for the glucuronoside fraction.

It was previously noted in control individuals that only a fraction of the total free radioactivity moved on paper chromatograms in the cortisol area (11, 13). The same phenomenon was observed during late pregnancy, as shown in Table II; the percentage of activity corresponding to cortisol was rather constant throughout the experiment and similar to that observed in a nonpregnant female (subject B. A.) and in normal young adult males (13). Even though no attempt was made to characterize the extra-cortisol radioactivity, it is reasonable to believe that area No. 4 of the chromatograms would correspond to

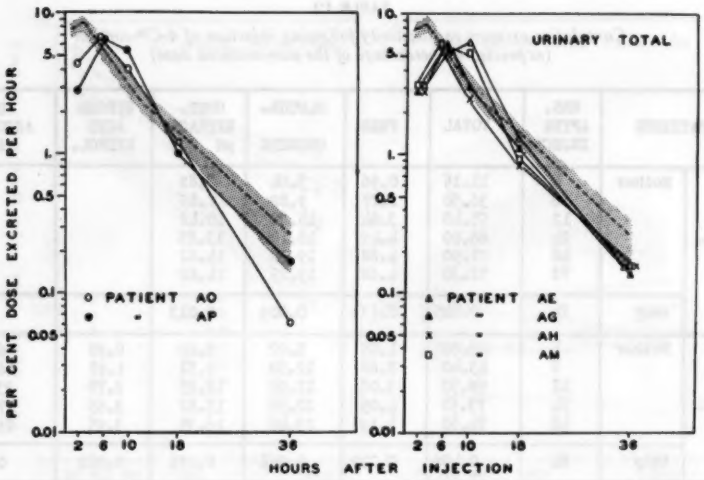


FIG. 4. RATES OF TOTAL URINARY EXCRETION OF RADIOACTIVITY IN MOTHERS WHO RECEIVED 4-C^{14}-CORTISOL 24 AND 48 HOURS PRIOR TO AN ELECTIVE, REPEAT CESAREAN SECTION (LEFT) AND IN MOTHERS WHO RECEIVED 4-C^{14}-CORTISOL 17 TO 69 MINUTES PRIOR TO SECTION (RIGHT)

The broken line joins the average of the rates obtained in five normal males (13) and two normal non-pregnant females (15). The shaded area shows the range of values for these normal individuals. The full lines join the values obtained in the patients studied.

the dihydro-compounds, while areas Nos. 1 and 2 would correspond to the 20-hydroxylated metabolites of cortisol as well as the tetrahydro-compounds.

Radioactivity in the urine of mothers and their babies

The activities of the various urinary fractions are recorded in Table III. The rate of excretion of radioactivity per hour was calculated for the individual fractions covering different periods of urinary collection. These values were plotted on semi-logarithmic paper and the results are shown in Figures 4 to 7. These figures show also the average values obtained in seven normal subjects (13, 15) with the range of variation of these values.

The total 48-hour excretion of radioactivity by the various patients was smaller than that of the normal subjects of previous studies (13, 15) who excreted an average of 87 per cent of the dose. This was mainly due to a decreased rate of excretion during the first 4 hours following 4-C^{14}-cortisol administration (Figure 4). Excretion from the fourth to the twelfth hour was equal to, and in some cases higher than, that seen in controls. After the twelfth hour the values were at the lower limit of normal.

The urinary "free fraction" was within the normal range during the first 4 hours (Figure 5), but from the fourth to the twelfth hour, the rate of excretion in patients studied was three to five times higher than found in control subjects.

Conversely, the amount of radioactivity in the urinary "glucuronoside fraction" was lower than average during the first 8 hours after 4-C^{14}-cortisol injection (Figure 6), following which the values approached the lower limits of the control range.

The amount of radioactivity obtained after 48-hour continuous ether extraction at pH 0.8 was somewhat larger than the normal values during the 4 to 8 hour collection period (Figure 7). Thereafter, the rate of excretion was variable.

Table IV shows the difference between the total urinary radioactivity and the sum of the activities found in the "free," "glucuronoside" and "continuous extraction" fractions. Following the removal of these various fractions, the urine residue

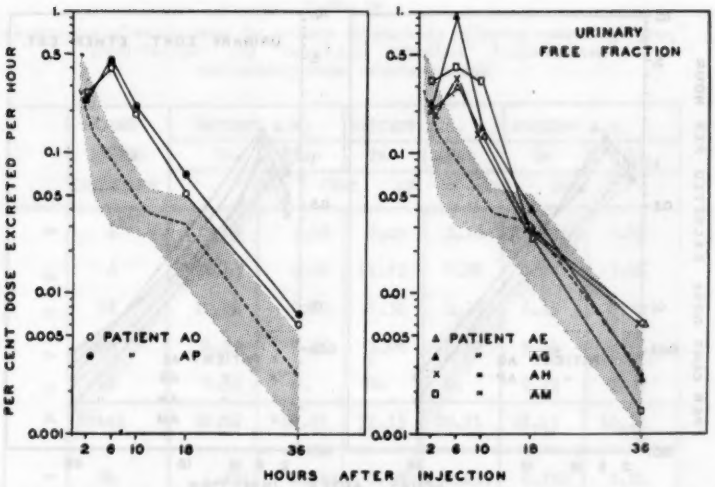


FIG. 5. RATES OF URINARY EXCRETION OF RADIOACTIVITY READILY EXTRACTABLE BY CHLOROFORM IN MOTHERS WHO RECEIVED 4-C^{14}-CORTISOL 24 AND 48 HOURS PRIOR TO ELECTIVE, REPEAT CESAREAN SECTION (LEFT) AND IN MOTHERS WHO RECEIVED 4-C^{14}-CORTISOL 17 TO 69 MINUTES PRIOR TO SECTION (RIGHT)

See legend for Figure 4.

of patients A. M. and A. O. still contained 21.27 and 20.71 per cent of the dose, respectively. The difference between these two values and the total radioactivity unaccounted for (approximately 10 per cent of dose) probably represents the sum of the experimental errors (handling and counting

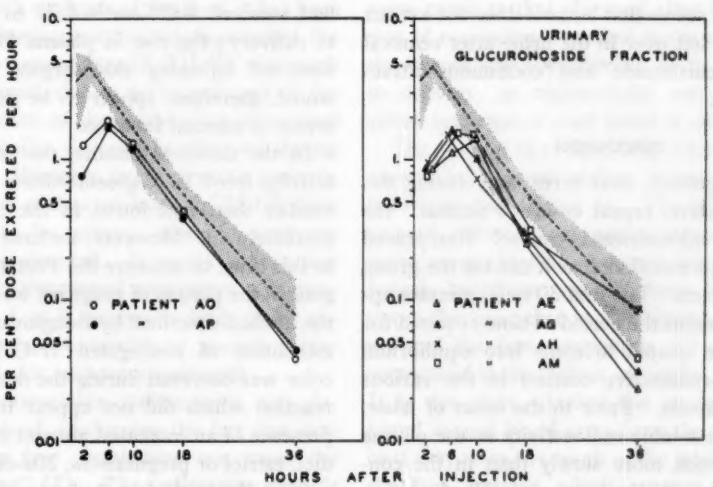


FIG. 6. RATES OF URINARY EXCRETION OF RADIOACTIVITY EXTRACTABLE BY CHLOROFORM FOLLOWING β-GLUCURONIDASE HYDROLYSIS IN MOTHERS WHO RECEIVED 4-C^{14}-CORTISOL 24 AND 48 HOURS PRIOR TO ELECTIVE, REPEAT CESAREAN SECTION (LEFT) AND IN MOTHERS WHO RECEIVED 4-C^{14}-CORTISOL 17 TO 69 MINUTES PRIOR TO SECTION (RIGHT)

See legend for Figure 4.

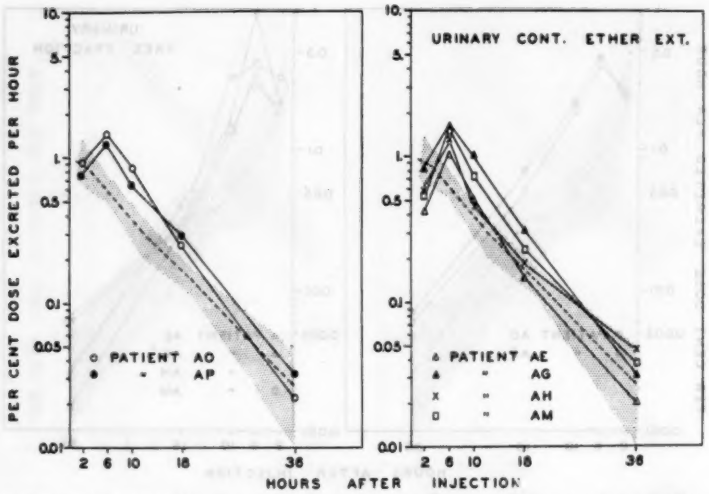


FIG. 7. RATES OF URINARY EXCRETION OF RADIOACTIVITY OBTAINED BY 48-HOUR CONTINUOUS ETHER EXTRACTION AT pH 0.8 IN MOTHERS WHO RECEIVED 4-C^{14}-CORTISOL 24 AND 48 HOURS PRIOR TO ELECTIVE, REPEAT CESAREAN SECTION (LEFT) AND IN MOTHERS WHO RECEIVED 4-C^{14}-CORTISOL 17 TO 69 MINUTES PRIOR TO SECTION (RIGHT)

See legend for Figure 4.

of each individual urine sample) made up to that point. In patient A. G., the butanol extract of the urine residue contained only 10.1 per cent of the dose and it seems that butanol does not extract all the activity left over in the urine after removal of "free," "glucuronoside" and "continuous extraction" fractions.

DISCUSSION

During pregnancy, near term, and during delivery by "elective, repeat cesarean section," the intravenously administered cortisol disappeared rapidly from the circulation as it did for the group of control subjects. The first 30 to 60 minutes appeared to represent the period of time required for the radioactive steroid to come into equilibrium with the non-radioactive cortisol in the various body compartments. Prior to the onset of labor, the chloroform-soluble radioactivity in the plasma disappeared much more slowly than in the control subjects despite the increase in blood volume observed in pregnant women (16). These findings demonstrated a rather marked modification in the metabolism of 4-C^{14}-cortisol. When the surgical stress associated with the cesarean section intervened shortly after the administration of radioactive steroid (69 minutes or less), the curves of the "free" and "glucuronoside" fractions in plasma were similar to those found in patients who had received 4-C^{14}-cortisol 24 to 48 hours prior to delivery; the rise in plasma 17-OH-CS levels observed following this surgical procedure (6) would, therefore, appear to be due to a true increase in adrenal function.

In the maternal plasma, the amount of radioactivity freed by β-glucuronidase hydrolysis was smaller than that found in the controls but not absolutely nil. However, we have been unable, up to this time, to measure the Porter-Silber chromogens in the plasma of pregnant women when using the method described by Bongiovanni (17) for the estimation of conjugated 17-OH-CS. A pink color was observed during the development of the reaction which did not appear to be due to the presence of an increased amount of estrone, estradiol, estriol or pregnane-3α, 20α-diol in the plasma extract obtained after β-glucuronidase hydrolysis. Our inability to measure the conjugated 17-OH-CS by the Porter-Silber reaction must be due, therefore, to interference by some other material rather than the total absence of the glucuronoside of the 17-OH-CS.

TABLE IV

Comparison of unaccounted for urinary radioactivity following removal of "free," "glucuronoside" and "continuous extraction" fractions and radioactivity found in urine residue

	HOURS AFTER INJECTION	PATIENT A.M.		PATIENT A.O.		PATIENT A.G.	
		I*	II**	I*	II**	I*	III***
		PER CENT OF THE DOSE					
MOTHER	4	6.05	3.48	7.00	3.76	5.73	2.45
	8	10.44	6.62	11.71	7.78	8.68	3.84
	12	10.44	7.10	7.36	4.25	6.15	2.32
	24	4.52	4.05	5.06	4.92	5.44	1.18
	48	0.57	0.	0.	0.	2.19	0.31
	Total	32.02	21.27	31.13	20.71	28.19	10.10
CORD	24	1.287	1.033	0.260	0.139	0.750	0.24
	48	0.284	0.200	—	—	0.477	0.14
	Total	1.571	1.233	0.260	0.139	1.227	0.38

* Difference between total urinary radioactivity and the sum of activities in "free," "glucuronoside" and "continuous extraction" fractions.

** Radioactivity in urine residue following removal of "free," "glucuronoside" and "continuous extraction" fractions.

*** Radioactivity in butanol extract of urine residue following removal of "free," "glucuronoside" and "continuous extraction" fractions.

In patients with cirrhosis of the liver, it has been shown that following cortisol administration the rate of disappearance of free 17-OH-CS was much slower than normal, due to an impairment of the rate of conjugation of the metabolites of cortisol (18, 19). A similar situation is observed during late pregnancy. However, in the cirrhotic patients, the resting levels of free plasma 17-OH-CS have been reported to be normal, while in pregnant women they are elevated. It would appear that during pregnancy the control mechanism of homeostasis is impaired, as it appears to be in dying patients (20). It is not possible at present to explain the reasons for such an impairment.

Chloroform extractable radioactivity was detected in cord blood, confirming the fact that cortisol and/or its free metabolites can cross the "placental barrier" (7). The radioactive steroids appeared to reach the fetus very rapidly since they were found in cord plasma of baby A. G. 17 minutes after the injection of 4-C^{14}-cortisol into the mother. It was interesting to note that the ratio of maternal to cord free radioactivity was in the same range as that observed after the administration of non-isotopic cortisol to the mother. When the steroid was administered 24 to 48 hours prior to delivery, no radioactivity was measurable in either maternal or cord blood at delivery time.

The amount of radioactivity liberated from cord plasma by β-glucuronidase hydrolysis was small but significant (Table I). The ratio of maternal to cord values of conjugated steroids was in the same range as that observed for the free compounds but there was no direct relationship between the two; that is, the highest values for the ratio of the free compounds did not necessarily correspond to the highest values for the conjugates. It is not clear whether the conjugated steroids found in cord blood came from the mother as such or if they were the result of the fetal metabolism of the free steroids which crossed the "placental barrier."

The fact that no radioactivity, free or conjugated with glucuronic acid, was found in the cord blood 24 to 48 hours after the administration of 4-C^{14}-cortisol to the mother would suggest that

the fetus has means of disposing of cortisol and its metabolites. They could either be excreted into the amniotic fluid or returned to the maternal circulation.

In the present study it was observed that the total amount of urinary radioactivity excreted by the mothers was significantly smaller than that eliminated by normal individuals. Urine collections were completed prior to the surgical procedure only in subject A. P. In subject A. O., only the first 24-hour collection was terminated prior to surgery. From these limited data, it would appear that during pregnancy, near term, the rate of excretion of total radioactivity following 4-C^{14}-cortisol administration is somewhat slower than in normal individuals.[4] However, the total urinary excretion represents the sum of various groups of metabolites and it was found that the "glucuronoside fraction" only was markedly diminished while the "free fraction" was increased; since the glucuronoside fraction constitutes a much larger percentage of the total, it is understandable that the total urinary radioactivity would be decreased.

We have already mentioned above that, following 4-C^{14}-cortisol administration to the mothers, the half-life of free radioactivity in plasma was almost twice that of normal, and the total amount of urinary radioactivity was probably smaller than normal. At the same time, the plasma levels of free 17-OH-CS during pregnancy, near term, and prior to the onset of labor were found to be not more than twice the 8 to 9 A.M. levels of control subjects. It would appear, therefore, that there is no real increase in the production of adrenocortical steroids in pregnancy, but rather a decreased rate of catabolism of the corticosteroids produced.[5] It would seem to follow that the urinary excretion of corticosteroids during pregnancy, near term, should not be different from that of non-pregnant adult females; this is in contradiction to the interpretation of most results reported to date in the literature. An increased urinary excretion of glucocorticoids during pregnancy has been reported by Venning (22). Heard, Sobel and Venning (23), Jailer (24), and Devis (25) have noted an increased excretion of neutral reducing lipids. An elevation in the urinary levels of formaldehydrogenic substances and the steroids reacting with dinitrophenylhydrazine has also been reported by Tobian (26), and by Jayle, Desgrez, Serpicelli, and Rozeg (27), respectively. However, Gray (28) found that free cortisone excretions were higher than normal while the concentration of tetrahydrocortisone, obtained only after hydrolysis, was in the control range or perhaps slightly lower. Our data tend to support his findings and those of Devis (25) who reported a marked increase in the free 17-hydroxycorticoids in the urine of pregnant women. In the bioassay employed by Venning, Kazmin, and Bell (29), the free corticosteroids are not removed prior to pH 1.0 hydrolysis and it is probable that cortisol and cortisone are biologically more potent than their tetrahydro derivatives. It is conceivable, therefore, that the increase in glucocorticoid concentration observed by Venning (22) might be related to the increased proportion of free compounds excreted in the terminal phase of pregnancy. The methods which measure neutral reducing lipids or formaldehydogenic steroids are known to be relatively nonspecific. It is possible that some of the other steroids and/or their metabolites which are produced and excreted in large amounts during pregnancy might interfere with the measurement of urinary corticosteroids by these techniques. On the other hand, the determination of urinary 17,21-dihydroxy-20-ketones by the reaction of Porter and Silber (30) is probably somewhat more specific; little or no increase in urinary corticoids was observed during pregnancy when using this technique (25, 27). Little change in 17-ketogenic steroid excretion was also reported (31).

If we assume that the newborn disposes of the radioactive steroids received from the mother in the same manner that he does following the intravenous administration of a given dose of 4-C^{14}-cortisol, the total amount of urinary radioactivity excreted in the first 48 hours following birth would represent approximately 80 per cent of the total radioactivity which crossed the placental barrier; that is, 2 to 3 per cent of the dose administered to the mother. However, this assumption might

[4] Similar results have been obtained in two other patients (subjects A. T. and A. U.) who received 4-C^{14}-cortisol 48 hours prior to surgery (21).

[5] The same conclusion was drawn by Mills (31) from his work on plasma free cortisol levels and 17-ketogenic steroid excretion during pregnancy.

not be entirely true since it is possible that maternal conjugated steroids cross the "placental barrier," and because it is also probable that free and/or conjugated steroids are returned from the fetus to the mother, the entire process being a dynamic phenomenon.

When each urinary fraction excreted by the baby was expressed in percentage of the total output of radioactivity, the values for the glucuronoside fraction ranged from 3.9 to 20 per cent of the total with an average of 12 per cent, while the average for 7 normal adults was 51.6 per cent and that of the mothers studied was 27.5 per 'cent. The newborn appeared to have a diminished ability to conjugate cortisol metabolites with glucuronic acid. The pattern of the other urinary fractions did not differ greatly from that observed in normal adults, and consequently the amount of urinary radioactivity unaccounted for following the various hydrolyses was very large. As seen in Table III, a large amount of this activity was left in the urine and even butanol did not appear to remove it completely. The same phenomenon was observed in normal adults but to a lesser degree. It would seem that there are modes of conjugation of steroids with compounds other than sulfuric acid, and it is possible that the newborn, because of his partial inability to conjugate with glucuronic acid, would use these other modes of detoxification to a larger extent than does the normal adult.

The sum of the amounts of radioactivity excreted by the mother and her baby appeared to be somewhat smaller than the total urinary excretion observed in control subjects. We have some indication that some of this activity was trapped in the placenta and that some of it could be detected in amniotic fluid. It must also be noted that when 4-C^{14}-cortisol was administered shortly prior to the surgical procedure, a certain amount of the hormone was contained in the 500 to 750 ml. of blood lost by the mother at the time of delivery. Another possibility is that the mother might excrete amounts of fecal radioactivity larger than normal. Further work is in progress to study this problem.

SUMMARY

The metabolism of 4-C^{14}-cortisol during pregnancy, near term, was found to be markedly altered. In plasma, the half-life of free radioactivity was twice that observed in control subjects and the amount of radioactivity released by β-glucuronidase hydrolysis was smaller than normal. The total amount of urinary radioactivity excreted was diminished, with an increase in the "free fraction" and a decrease in the "glucuronoside fraction." These data suggest that the increase in plasma free 17-hydroxycorticosteroids observed near term is related to a slower rate of catabolism of cortisol rather than to a more rapid rate of its production.

Similar results were obtained when the surgical procedure of elective, repeat cesarean section was performed 17 to 69 minutes after the administration of the radioactive steroid. The increase in plasma free 17-hydroxycorticosteroids observed following this procedure would then appear to be related to a real increase in cortisol production rather than solely to an interference in its catabolism.

In cord plasma, the amounts of free radioactivity and that released by β-glucuronidase hydrolysis were one-half to one-fifth those of the corresponding mother; this confirmed the observation that cortisol and/or its free metabolites can cross the "placental barrier."

No radioactivity, free or conjugated with glucuronic acid, was found in cord plasma 24 to 48 hours after 4-C^{14}-cortisol administration to the mother; this suggested that the fetus has means of disposing of cortisol and its metabolites.

The total amount of radioactive steroid crossing the placenta appeared to represent 2 to 3 per cent of the dose administered to the mother. However, this might be too low a figure if some of the steroids are returned from the fetus to the mother.

ACKNOWLEDGMENTS

The authors are indebted to Drs. Lawson Wilkins and Nicholson J. Eastman for their great interest in this work.

REFERENCES

1. Gemzell, C. A., Blood levels of 17-hydroxycorticosteroids in normal pregnancy. J. Clin. Endocrinol. & Metab., 1953, 13, 898.
2. Morris, C. J. O. R., and Williams, D. C., Estimation of individual adrenocortical hormones in human peripheral blood *in* Ciba Foundation Colloquia on Endocrinology. London, J. & A. Churchill Ltd., 1953, vol. 7, p. 261.

3. Bayliss, R. I. S., Browne, J. C. McC., Round, B. P., and Steinbeck, A. W., Plasma-17-hydroxycorticosteroids in pregnancy. Lancet, 1955, 1, 62.
4. Robinson, H. J., Bernhard, W. G., Grubin, H., Wanner, H., Sewekow, G. W., and Silber, R. H., 17,21-dihydroxy-20-ketosteroids in plasma during and after pregnancy. J. Clin. Endocrinol. & Metab., 1955, 15, 317.
5. Assali, N. S., Garst, J. B., and Voskian, J., Blood levels of 17-hydroxycorticosteroids in normal and toxemic pregnancies. J. Lab. & Clin. Med., 1955, 46, 385.
6. Migeon, C. J., Keller, A. R., and Holmstrom, E. G., Dehydroepiandrosterone, androsterone and 17-hydroxycorticosteroid levels in maternal and cord plasma in cases of vaginal delivery. Bull. Johns Hopkins Hosp., 1955, 97, 415.
7. Migeon, C. J., Prystowsky, H., Grumbach, M. M., and Byron, M. C., Placental passage of 17-hydroxycorticosteroids: comparison of the levels in maternal and fetal plasma, and effect of ACTH and hydrocortisone administration. J. Clin. Invest., 1956, 35, 488.
8. Gemzell, C. A., Variations in plasma levels of 17-hydroxycorticosteroids in mother and infant following parturition. Acta endocrinol., 1954, 17, 100.
9. Klein, R., Fortunato, J., and Papadatos, C., Free blood corticoids in the newborn infant. J. Clin. Invest., 1954, 33, 35.
10. Hellman, L., Bradlow, H. L., Adesman, J., Fukushima, D. K., Kulp, J. L., and Gallagher, T. F., The fate of hydrocortisone-4-C^{14} in man. J. Clin. Invest., 1954, 8, 1106.
11. Peterson, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B., and Bunim, J. J., The physiological disposition and metabolic fate of hydrocortisone in man. J. Clin. Invest., 1955, 34, 1779.
12. Peterson, R. E., and Wyngaarden, J. B., The miscible pool and turnover rate of hydrocortisone in man. J. Clin. Invest., 1956, 35, 552.
13. Migeon, C. J., Sandberg, A. A., Decker, H. A., Smith, D. F., Paul, A. C., and Samuels, L. T., Metabolism of 4-C^{14}-cortisol in man: Body distribution and rate of conjugation. J. Clin. Endocrinol. & Metab., 1956, 16, 1137.
14. Bush, I. E., Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. Biochem. J., 1952, 50, 370.
15. Migeon, C. J., Unpublished data.
16. Gemzell, C. A., Robbe, H., and Sjöstrand, T., Blood volume and total amount of haemoglobin in normal pregnancy and the puerperium. Acta obst. et gynec. Scandinav., 1954, 33, 289.
17. Bongiovanni, A. M., Detection of corticoid conjugates in human blood. J. Clin. Endocrinol. & Metab., 1954, 14, 341.
18. Brown, H., Willardson, D. G., Samuels, L. T., and Tyler, F. H., 17-hydroxycorticosteroid metabolism in liver disease. J. Clin. Invest., 1954, 33, 1524.
19. Bongiovanni, A. M., Eberlein, W. R., Grumbach, M. M., Van Wyk, J. J., and Clayton, G., Conjugates of adrenal corticoids in human plasma. Proc. Soc. Exper. Biol. & Med., 1954, 87, 282.
20. Sandberg, A. A., Eik-Nes, K., Migeon, C. J., and Samuels, L. T., Metabolism of adrenal steroids in dying patients. J. Clin. Endocrinol. & Metab., 1956, 16, 1001.
21. Migeon, C. J., Bertrand, J., Wall, P. E., Stempfel, R. S., and Prystowsky, H., Metabolism and placental transmission of cortisol during pregnancy, near term *in* Ciba Foundation Colloquia on Endocrinology. London, J. & A. Churchill Ltd., 1957, In press.
22. Venning, E. H., Adrenal function in pregnancy. Endocrinology, 1946, 39, 203.
23. Heard, R. D. H., Sobel, H., and Venning, E. H., The neutral lipide-soluble reducing substances of urine as an index of adrenal cortical function. J. Biol. Chem., 1946, 165, 699.
24. Jailer, J. W., Adrenal function during pregnancy and the effect of ACTH during pregnancy *in* Proc. Second Clinical ACTH Conference. New York, The Blakiston Co., 1951, vol. 1, p. 77.
25. Devis, R., L'élimination des corticoïdes au cours de la grossesse. Gynéc. et obst., 1954, 53, 57.
26. Tobian, L., Jr., Cortical steroid excretion in edema of pregnancy, pre-eclampsia and essential hypertension. J. Clin. Endocrinol., 1949, 9, 319.
27. Jayle, M.-F., Desgrez, P., Serpicelli, J., and Rozeg, J., Dosage des corticoïdes urinaires après hydrolyse biologique. Ann. d'endocrinol., 1953, 14, 877.
28. Gray, C., Excrétion des hormones chez la femme diabétique enceinte. Ann. d'endocrinol., 1954, 15, 22.
29. Venning, E. H., Kazmin, V. E., and Bell, J. C., Biological assay of adrenal corticoids. Endocrinology, 1946, 38, 79.
30. Porter, C. C., and Silber, R. H., A quantitative color reaction for cortisone and related 17,21-dihydroxy-20-ketosteroids. J. Biol. Chem., 1950, 185, 201.
31. Mills, I. H., Adrenal steroid metabolism: studies in pregnancy in relation to the general problem. M.D. Thesis, University of Cambridge, 1956.

A SIMPLE NEPHELOMETRIC METHOD FOR THE DETERMINATION OF HUMAN SERUM BETA-LIPOPROTEIN [1]

By PETER BERNFELD, MORDECAI E. BERKOWITZ, AND VIRGINIA M. DONAHUE

(From the Department of Biochemistry and Nutrition, and the Cancer Research and Cancer Control Unit of the Department of Surgery, Tufts University School of Medicine, Boston, Mass.)

(Submitted for publication December 3, 1956; accepted May 23, 1957)

The measurement of lipoproteins in human serum is generally carried out by one of the following techniques: ultracentrifugal flotation (1–4); zone electrophoresis and subsequent staining of the lipids (5, 6); electrophoresis followed by cholesterol and/or phospholipid determinations on eluates of the supporting medium (7–9); and fractional precipitation of the serum proteins by ethanol at low salt concentrations, followed by lipid analyses of the fractions (10–13).

In the present paper, a new method of determination will be described which is specific for β-lipoprotein. This new method is distinguished from the above-mentioned ones by its marked simplicity and rapidity. It is based on a new principle: the fact that β-lipoprotein is the only protein of human serum which is precipitated by sulfated amylopectin at pH 8.6 (14, 15). At high dilutions of the serum, the resulting precipitate is highly dispersed, and its quantity can be estimated nephelometrically.

METHOD

Reagents. Sulfated amylopectin was obtained as the potassium salt (with 15 to 17 per cent S), after treatment of corn amylopectin for five hours at 70° C. with chlorosulfonic acid in pyridine (16). Amylopectin was prepared by the method of Schoch (17) and care was taken to remove all amylose. "Tris" buffer [2] of pH 8.6 at 23° C. and of ionic strength $\mu = 0.05$ was prepared according to Gomori (18).

Procedure. Serum is obtained by centrifugation of clotted blood in a clinical centrifuge at 2,000 rpm. Only markedly turbid sera have to be cleared, *e.g.*, by centrifuging them for twenty minutes in a high speed attachment of an International centrifuge at 20,000 rpm.[3] An aliquot of 0.1 ml. of serum is diluted to 25 ml. with Tris buffer in a volumetric flask, and the turbidity of this solution is measured in a ¾-inch fused black cell of a Fisher Nefluoro-Photometer. The solution is poured back into the volumetric flask and 0.15 ml. of a 0.1 per cent solution of sulfated amylopectin are added. After mixing the contents, the turbidity is determined in the same cell as before.

The instrument is adjusted to 100 per cent transmittance by the use of a ¾-inch fused black cell containing a freshly prepared turbidity reference solution. The latter is obtained by mixing 10 ml. of 0.16 mM K_2SO_4, 2 ml. of 2N HCl and 2 ml. of 0.5 M $BaCl_2$ containing also 1 Gm. of polyvinylalcohol [4] per 100 ml. to maintain the $BaSO_4$ in suspension. Since the $BaSO_4$ slowly deposits in spite of the polyvinylalcohol, this solution cannot be used as a reference for longer periods of time than one hour after it has been prepared. Solutions of various high polymers can be advantageously used as stable turbidity standards, after they have been compared to a freshly prepared $BaSO_4$ standard. A 1 per cent solution of PVM/MA, half amide, type 30,[5] is well suited for this purpose since it does not change its turbidity for long periods of time.

The scattered light due to the turbidity is measured by reading the dial scale, termed per cent transmittance, and the concentration of β-lipoprotein is expressed as the difference in turbidity between the readings with and without added sulfated amylopectin. This value is called the *nephelometric index* of β-lipoprotein.[6] A correction can be made for the turbidity of the sulfated amylopectin which is, however, very small. Recent experiments carried out with the Coleman Nephelometer using commercial nephelos standards have given excellent results.

[1] This investigation was supported in part by research grant H-2025 from the National Institutes of Health, Public Health Service, and by an Institutional Grant of the American Cancer Society, Inc., New York, New York.

[2] Trishydroxymethylaminomethane, purchased as Sigma 7–9 from Sigma Chemical Company, St. Louis, Missouri.

[3] It has been found that centrifugation of normally clear serum at 20,000 rpm for 45 minutes does not alter the results obtained with the method described herein.

[4] Elvanol, duPont, grade 71–24.

[5] A copolymer of methyl vinyl ether and maleic anhydride, manufactured by The General Aniline and Film Corp., New York, New York.

[6] Symbols used in the statistical evaluations have been taken from Dixon, W. J., and Massey, F. J., An Introduction to Statistical Analysis. New York, McGraw-Hill & Co., 1951.

EXPERIMENTAL

Specificity of the reaction

Previous experiments using the moving boundary technique of electrophoresis have clearly shown that among all serum proteins only β_1-globulin is precipitated by sulfated amylopectin (15). Additional evidence indicating the specificity of this reaction for β_1-globulin was obtained by an independent method. When human serum was fractionated by zone electrophoresis in a medium of starch granules, according to Kunkel and Slater (9), only the fractions with the mobility of β_1-globulin were found to produce turbidities with sulfated amylopectin (see Figure 1). All other fractions containing albumin, α_1-, α_2-, β_2- and γ-globulin did not react more appreciably with the polysulfate ester than the eluates containing no protein (fractions -8 to -3 and 16 to 21). Further characterization of the precipitated β_1-globulin has revealed its lipoprotein nature with a ratio of lipide to polypeptide content (on a weight basis) of about 3 to 1 (15). The precipitated serum protein can, therefore, be considered to be β-lipoprotein.

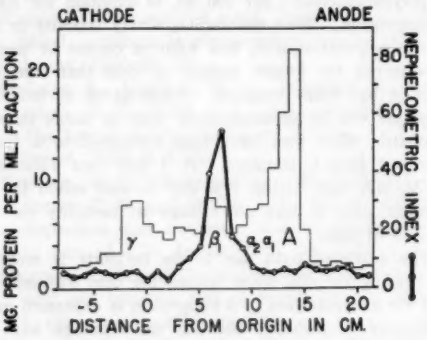


FIG. 1. NEPHELOMETRIC ANALYSES OF 18 FRACTIONS OBTAINED FROM HUMAN SERUM BY ZONE ELECTROPHORESIS

Electrophoresis of 1.6 ml. of normal human serum in a square cuvette of 4.5 cm.² sectional area, using a supporting medium of potato starch granules, and Tris-citrate buffer of pH 8.6 and ionic strength 0.06, electric field 3.3 volts per cm. during 22 hours. One-cm. wide sections of the medium were eluted with 3 ml. of 0.9 per cent saline. Protein analyses (bar diagram) were performed on 0.1-ml. aliquots according to Lowry, Rosebrough, Farr, and Randall (19), A, α_1, α_2, β_1 and γ being the portions of albumin and the corresponding globulins. The apparent cathodic migration of γ-globulin was due to electro-osmotic flow. The nephelometric analyses (heavy line) were carried out on 2 ml.-aliquots in the presence of 300 γ crystalline bovine serum albumin to stabilize β-lipoprotein.

Stability of the turbidity

Under the conditions of the procedure, the finely dispersed precipitate of β-lipoprotein with sulfated amylopectin neither deposits nor floats to the surface. Consequently, it is immaterial whether the nephelometric reading is carried out immediately after the addition of the reagent, or later (see Table I).

Concentration of sulfated amylopectin

The concentration of sulfated amylopectin used in this procedure is 150 γ per 25 ml. of reaction mixture with 0.1 ml. of serum; a maximal turbidity is obtained at this concentration of sulfated amylopectin (see Figure 2). Under these conditions, slight errors in the amount of sulfated amylopectin added would produce only negligible errors in the resulting turbidity.

Nature of the precipitant

Among all substances tested, sulfated amylopectin yields the highest turbidities and, hence, the greatest sensitivity of the method. The degree of sulfation of amylopectin is without importance for the nephelometric index, provided that at least two of the three available hydroxyl groups of the repeating unit in amylopectin are sulfated. Slightly lower turbidity values are obtained when the reagent is contaminated with sulfated amylose. This happens when amylose was not completely removed from amylopectin before sulfation.

TABLE I

Stability of the turbidity obtained after addition of sulfated amylopectin to serum

Subject number	Nephelometric index, read at different time intervals after the addition of sulfated amylopectin to serum		
	After 15 min.	After 75 min.	After 24 hours
1	52.5	52	51
2	44	46.5	46.5
3	48	47	45
4	51.5	52.5	52.5
5	48	47	47

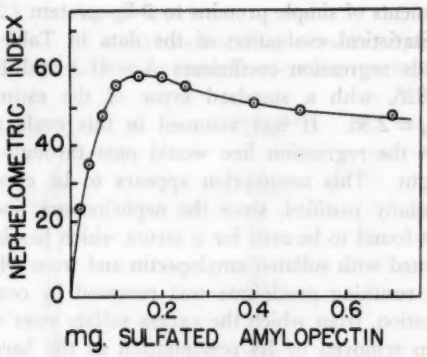


FIG. 2. RELATIONSHIP OF NEPHELOMETRIC INDEX TO CONCENTRATION OF SULFATED AMYLOPECTIN, WHEN WHOLE SERUM IS USED AS THE SOURCE OF LIPOPROTEIN

Each sample contained 0.1 ml. of serum (from a single batch) and the amount of sulfated amylopectin indicated on the abscissa, in a total volume of 25 ml. Tris buffer. With the use of preparations of isolated β-lipoprotein, a maximum of the nephelometric index was found at 0.03 mg. sulfated amylopectin per sample.

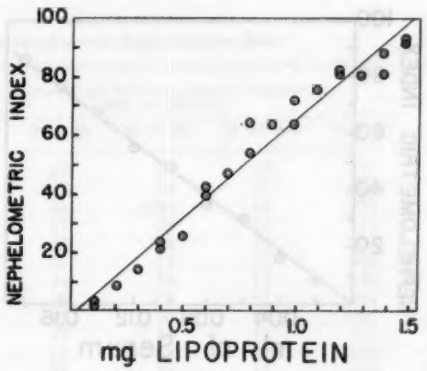


FIG. 3. RELATIONSHIP OF NEPHELOMETRIC INDEX TO CONCENTRATION OF β-LIPOPROTEIN

Each sample contained the amount of β-lipoprotein indicated on the abscissa, 0.3 mg. crystalline bovine serum albumin to stabilize the lipoprotein and 0.03 mg. sulfated amylopectin in a total volume of 25 ml. of Tris buffer. The concentration of β-lipoprotein was calculated by adding up the concentrations of total polypeptide (according to Lowry and co-workers [19]) and total lipide. This value was within 5 per cent of the dry weight of the preparation after exhaustive dialysis against distilled water. The solid line represents the estimated regression line.

Linearity of the reaction

The nephelometric index has been found to be proportional to the concentration of β-lipoprotein (see Figure 3). The lipoprotein used in this experiment was obtained by ultracentrifugation of normal human serum for 22 hours in a No. 40 head of the Spinco Model L ultracentrifuge at 40,000 rpm, average of 105,400 × G, in a saline density tube (20). Fractional withdrawal of the solution from the density gradient tube yielded a fraction (at a density of the solution of d = 1.030) containing lipoprotein with 70.5 per cent total lipides and a ratio of total cholesterol to phospholipide of 1.23 (on a weight basis), and which migrated as a single peak on moving boundary electrophoresis, with a mobility of 3.5 to 3.7×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}.

Sensitivity and precision of the method

According to the data in Figure 3, the method is sensitive to less than 100 γ of β-lipoprotein per sample. When preparations of isolated β-lipoprotein are assayed, the precision of the method is characterized by a standard error of estimate of 5.85, calculated from the data in Figure 3. With whole serum, the precision is much higher (see Figure 4), apparently because of a stabilization of the lipoprotein by its environment of serum proteins. Thus, a standard error of estimate of 1.61 is obtained from the data in Figure 4, with a confidence interval of $\pm$ 3.4 to $\pm$ 3.6 nephelometric units over the whole range of observation, for a level of significance of $\alpha = 5$ per cent.

Influence of inherent turbidity of serum

The inherent turbidity of the serum sample does not influence the nephelometric lipoprotein index, since the results have been found to be unchanged when turbid sera are cleared by centrifugation. It is recommended, however, to clear highly turbid serum samples in order to prevent a shifting of the turbidity readings into too high levels.

Comparison of the nephelometric index with electrophoretic and ultracentrifugal data

Table II shows the relationship between the turbidity obtained upon addition of sulfated amylopectin to serum at high dilutions (nephelometric index) and the amount of β_1-globulin precipitated at higher concentrations of serum. The latter

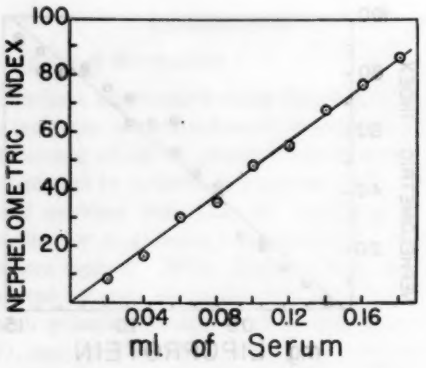


FIG. 4. RELATIONSHIP OF NEPHELOMETRIC INDEX TO AMOUNT OF SERUM

Each sample contained the amount of serum indicated on the abscissa and 0.15 mg. sulfated amylopectin in a total volume of 25 ml. Tris buffer.

data were calculated from the difference in absolute area measurements of the electrophoretic patterns (21) obtained with and without the addition of sulfated amylopectin. Due to the lipoprotein nature of the precipitate (15), a correction had to be made for the difference in refractive indices between simple proteins and lipoproteins of low densities (22). Thus, the protein concentration which would have been obtained for simple proteins has been multiplied by a factor of 1.09, representing the ratio of the refractive index increments of simple proteins to β-lipoprotein (23).

Statistical evaluation of the data in Table II yields regression coefficients A = 41.3 and B = 0.0826, with a standard error of the estimate $S_{y.x} = 2.36$. It was assumed in this evaluation that the regression line would pass through the origin. This assumption appears to be experimentally justified, since the nephelometric index was found to be zero for a serum which has been treated with sulfated amylopectin and from which the resulting precipitate was removed by centrifugation, from which the excess sulfate ester was then removed by its precipitation as the barium salt, and from which the excess of barium ion was finally removed by dialysis. Further statistical evaluation has shown that there is excellent agreement between these two methods. In fact, a t value of 6.31 was obtained in testing the hypothesis that there is *no* relationship; such a hypothesis would be acceptable only if t were between − 2.45 and 2.45 (level of significance $\alpha = 2.5$ per cent, d.f. = 6).

Data for lipoprotein analyses by the nephelometric method and by the ultracentrifugal flotation technique [7] are compared in Table III, and their statistical evaluation is presented in Table IV. No significant agreement between nephelometric measurements and any of the routinely computed ultracentrifugal Sf ranges can be observed.

TABLE II

Comparison of electrophoretic and nephelometric data on the effect of addition of sulfated amylopectin to serum

Subject number	Decrease in β-globulin from electrophoretic analyses*	Increase in turbidity (nephelometric index)†	Ratio of electrophoretic to nephelometric values
	(*mg. per 100 ml.*)		
1	490 ± 50‡	42	11.7 ± 1
2	490 ± 50	42	11.7 ± 1
3	480 ± 50	38	12.6 ± 1
4	640 ± 50	49.5	12.9 ± 1
5	490 ± 50	41	12.0 ± 1
6	390 ± 50	35.5	11.0 ± 1
7	510 ± 50	43.5	11.7 ± 1
8	490 ± 50	39	12.6 ± 1
Mean			12.0 ± 1

* Determined from the difference in absolute area measurements of the electrophoretic patterns, according to Tiselius and Kabat (21), and corrected for the refractive index increment of lipoglobulin (see text).

† For definition of nephelometric index, see text.

‡ Standard error of the measurement.

DISCUSSION

Quantitative interpretation of the nephelometric index

The new nephelometric method has been applied to whole serum as well as to isolated β-lipoprotein. The use of purified lipoprotein has presented certain difficulties, due mainly to the instability of lipoprotein preparations. These difficulties have been largely overcome by the addition of bovine serum albumin to the lipoprotein sample. On the basis of data obtained with isolated β-lipoprotein preparations (Figure 3), the nephelometric index would have to be multiplied by a factor of 14.65 in order to convert it into a value indicating β-lipoprotein in γ per sample,

[7] The ultracentrifugal experiments were performed by Dr. A. J. Barak, Department of Biochemistry, The University of Nebraska, College of Medicine, Omaha, Nebraska.

TABLE III

Comparison of nephelometric β-lipoprotein values to ultracentrifugal flotation data *

Subject number	Nephelometric index	Ultracentrifugal flotation values for lipoproteins (mg. per 100 ml.)				
		Sf 0–12	Sf 12–20	Sf 20–100	Sf 100–400	Sf 0–400
9	43	284	22	51	15	372
10	49.5	267	42	34	12	355
11	43	262	64	100	15	441
12	39	250	17	29	15	311
13	46	348	25	54	12	439
14	45	309	27	12	12	360
15	53.5	360	34	56	17	467
16	43	351	25	49	17	442
17	40	311	27	96	17	451
18	49.5	306	27	34	12	379
19	50.5	367	15	34	22	438
20	46.5	345	27	74	17	463

* The ultracentrifugal experiments were performed by Dr. A. J. Barak, Department of Biochemistry, University of Nebraska College of Medicine, Omaha, Nebraska.

and by the same factor to yield mg. of β-lipoprotein per 100 ml. of serum, provided a sample of 0.1 ml. of serum has been used for the analyses.

Another way of interpreting quantitatively the nephelometric index is the use of electrophoretic data presented in Table II in which the nephelometric index is compared to the decrease of β_1-globulin upon addition of sulfated amylopectin to serum. Thus, multiplication of the nephelometric index by 12.0, the mean value of the ratio of electrophoretic to nephelometric results, or by 12.1, the reciprocal of the regression coefficient (B = 0.0826), also yields a β-lipoprotein value in mg. per 100 ml. of serum.

Due to the marked heterogeneity of serum lipoproteins with respect to molecular weight, density, lipid content and lipid constituents, the various methods for their determination do not cover exactly the same range of individual lipoproteins and, hence, they do not yield identical results. In the light of these considerations, the discrepancy between the two factors of 14.65 and 12.1 is not significant. We tend, however, to give preference to the value of 12.1 since the factor of 14.65 has been obtained with the use of a lipoprotein preparation which may contain diverse impurities.

Sources of error

The main source of error in this procedure is the interference with extraneous turbid materials, *e.g.*, dust or traces of any water insoluble matter which might remain in the glassware after cleaning. For this reason, the above-described procedure provides that the two nephelometric readings, *i.e.*, with and without precipitant, are carried out on one single sample of diluted serum, rather than on two different aliquot portions. Thus, the

TABLE IV

Statistical comparison between nephelometric, flotation and electrophoretic data of lipoprotein analyses

Statistic	Sf—Range of flotation							Electrophoresis§
	0–12	12–20	0–20	20–100	0–100	100–400	0–400	
A*	45.7	45.7	45.7	45.7	45.7	45.7	45.7	41.3
B*	0.055	0.016	0.067	−0.046	0.025	0.113	0 024	0.0826
$S_{Y.x}$†	4.0	4.7	3.8	4.5	4.4	5.3	4.15	2.36
t‡	1.87	0.17	2.24	0.91	0.94	0.21	0.99	6.31

* Regression coefficients.

† Standard error of estimate.

‡ t value of statistical significance. No agreement exists between the data in each column and the nephelometric data if t is between −2.23 and +2.23 for the flotation values (α = 2.5 per cent, d.f. = 10), and for t between −2.45 and +2.45 for the electrophoretic data (α = 2.5 per cent; d.f. = 6).

§ From data in Table II.

presence of the same amount of an additional turbidity before and after the addition of precipitant does not affect the results, since only the difference between these two readings is important for the analysis of β-lipoprotein. The turbidity reference solution of barium sulfate is, however, subject to such extraneous factors, and it is recommended to compare at least eight individual reference solutions to each other, to eliminate those having a very high turbidity and to average the readings of the remainder of them. When stable reference solutions are used, *e.g.*, 1 per cent PVM/MA solution, as in routine determinations, their turbidity has to be compared to the barium sulfate standard only once. The use of commercial nephelos standards with the Coleman Nephelometer does not present these inconveniences.

Since fibrinogen also produces a turbidity with sulfated amylopectin, this plasma protein might interfere with the β-lipoprotein determinations in some pathological cases where the normal clotting of fibrinogen is impaired.

Specificity of the method

While there appears to be no doubt that the nephelometric method is specific for β_1-globulins (see Figure 1) and that that portion of the latter protein fraction which precipitates with sulfated amylopectin is a lipoprotein with over 70 per cent lipide content, it remains questionable whether the nephelometric method covers all of what is generally called β_1-lipoprotein or only a certain part of it. The latter assumption does not appear likely, however, since the nephelometric data in Table III, when multiplied by the factor 12.1, yield β-lipoprotein concentrations which are even higher than those found for the corresponding Sf 0-400 group.

Though it is generally believed that the β-lipoprotein fraction corresponds to the low density lipoproteins, as determined by ultracentrifugal flotation (24), no agreement between nephelometric index of β-lipoprotein and any of the four Sf groups of lipoproteins was found (see Tables III and IV), while the agreement between nephelometric index and the Sf 0-20 group appears to be barely significant. This lack of agreement is not surprising, however, since the delimitations between these Sf groups have been chosen arbitrarily. The increase in the t value from Sf 0-20 and its consequent decrease to Sf 0-12 would make it appear as if there existed another group of Sf, not routinely computed, which would more closely agree with the nephelometric data, *e.g.*, an arbitrary group of Sf 5-20.

A comparison of nephelometric lipoprotein index with total cholesterol values in about 30 subjects did not indicate a relation between these two factors. When the results of these two determinations were compared to each other in the same subject, however, remarkable parallelism was observed in many individuals.

SUMMARY

1. A new method for the determination of β-lipoprotein in human serum has been developed. It is based on the nephelometric measurement of a finely-dispersed precipitate of β-lipoprotein with sulfated amylopectin at pH 8.6.

2. This method is simple, rapid and requires as little as 0.1 ml. of serum. The sensitivity of the method is less than 100 γ of β-lipoprotein per sample, and the precision is given by a standard error of estimate of 1.61, when β-lipoprotein is determined in serum.

3. The specificity of this method for β-lipoprotein has been established by zone and moving boundary electrophoretic techniques.

4. No agreement between β-lipoprotein by nephelometry and ultracentrifugally-determined lipoprotein of any of the commonly computed flotation rate groups has been found. The nephelometric method is liable, however, to agree with a certain range of lipoproteins not commonly calculated, *e.g.*, Sf 5-20.

ACKNOWLEDGMENT

We are grateful to Dr. A. J. Barak for carrying out the ultracentrifugal study, and to Mrs. Sylvia Jacobson and Stuart Cook for valuable technical assistance.

REFERENCES

1. Gofman, J. W., Lindgren, F., Elliott, H., Mantz, W., Hewitt, J., Strisower, B., and Herring, V., The role of lipids and lipoproteins in atherosclerosis. Science, 1950, 111, 166.
2. Pedersen, K. O., On a low-density lipoprotein appearing in normal human plasma. J. Phys. Chem., 1947, 51, 156.
3. Havel, R. J., Eder, H. A., and Bragdon, J. H., The

distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest., 1955, **34**, 1345.

4. Hillyard, L. A., Entenman, C., Feinberg, H., and Chaikoff, I. L., Lipide and protein composition of four fractions accounting for total serum lipoproteins. J. Biol. Chem., 1955, **214**, 79.
5. Jencks, W. P., and Durrum, E. L., Paper electrophoresis as a quantitative method: The staining of serum lipoproteins. J. Clin. Invest., 1955, **34**, 1437.
6. Swahn, B., Studies on blood lipids. Scandinav. J. Clin. & Lab. Invest., 1953, **5**, Suppl. 9.
7. Langan, T. A., Durrum, E. L., and Jencks, W. P., Paper electrophoresis as a quantitative method: Measurement of alpha and beta lipoprotein cholesterol. J. Clin. Invest., 1955, **34**, 1427.
8. Fasoli, A., Electrophoresis of serum lipoproteins on filter-paper. Acta med. Scandinav., 1953, **145**, 233.
9. Kunkel, H. G., and Slater, R. J., Zone electrophoresis in a starch supporting medium. Proc. Soc. Exper. Biol. & Med., 1952, **80**, 42.
10. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K., and Uroma, E., A system for the separation of the components of human blood: Quantitative procedures for the separation of the protein components of human plasma. J. Am. Chem. Soc., 1950, **72**, 465.
11. Oncley, J. L., Gurd, F. R. N., and Melin, M., Preparation and properties of serum and plasma proteins. XXV. Composition and properties of human serum β-lipoprotein. J. Am. Chem. Soc., 1950, **72**, 458.
12. Russ, E. M., Eder, H. A., and Barr, D. P., Protein-lipid relationships in human plasma. I. In normal individuals. Am. J. Med., 1951, **11**, 468.
13. Herbst, F. S. M., Lever, W. F., Lyons, M. E., and Hurley, N. A., Effects of heparin on the lipoproteins in hyperlipemia. An electrophoretic study of the serum alpha and beta lipoproteins after their separation by fractionation of the plasma proteins or ultracentrifugal flotation. J. Clin. Invest., 1955, **34**, 581.
14. Bernfeld, P., and Nisselbaum, J. S., Reaction of human serum β-lipoglobulin with macromolecular polysulfate esters. Federation Proc., 1956, **15**, 220.
15. Bernfeld, P., Donahue, V. M., and Berkowitz, M. E., Interaction of human serum β-lipoglobulin with polyanions. J. Biol. Chem., 1957, **226**, 51.
16. Tipson, R. S., Sulfonic esters of carbohydrates. Advances in Carbohydrate Chem., 1953, **8**, 107.
17. Schoch, T. J., The fractionation of starch. Advances in Carbohydrate Chem., 1945, **1**, 247.
18. Gomori, G., Buffers in the range of pH 6.5 to 9.6. Proc. Soc. Exper. Biol. & Med., 1946, **62**, 33.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Folin phenol reagent. J. Biol. Chem., 1951, **193**, 265.
20. Oncley, J. L., and Mannick, V. G., Studies on human plasma lipoproteins. Trans. of 5th International Congress of Blood Transfusion, Paris, 1954, 890–896.
21. Tiselius, A., and Kabat, E. A., An electrophoretic study of immune sera and purified antibody preparations. J. Exper. Med., 1939, **69**, 119.
22. Hanig, M., and Shainoff, J. R., Specific refractive increment of serum lipoproteins obtained by flotation. J. Biol. Chem., 1956, **219**, 479.
23. Armstrong, S. H., Budka, M. J. E., Morrison, K. C., and Hasson, M., Preparation and properties of serum and plasma proteins. XII. The refractive properties of the proteins of human plasma and certain purified fractions. J. Am. Chem. Soc., 1947, **69**, 1747.
24. Jones, H. B., Gofman, J. W., Lindgren, F. T., Lyon, T. P., Graham, D. M., Strisower, B., and Nichols, A. V., Lipoproteins in atherosclerosis. Am. J. Med., 1951, **11**, 358.

INFLUENCE OF CORTISONE AND PREDNISOLONE ON HYPERBILIRUBINEMIA

By RICARDO KATZ, HÉCTOR DUCCI, AND HERNÁN ALESSANDRI

(From the Department of Medicine, University of Chile Medical School, Hospital Salvador, Santiago, Chile)

(Submitted for publication November 26, 1956; accepted May 16, 1957)

Our experience (1), as well as that of others (2–4), in the treatment of acute hepatitis with steroids, has shown as the most striking effect a rapid drop of serum bilirubin. This is accompanied by the clearing of clinical jaundice.

Solem and Olsen (5) have studied the influence of ACTH on the icteric index in cases of hepatitis and of post-hepatic jaundice. In the former, it dropped very rapidly but in the latter it was not significantly influenced. They believed that the different response of both types of jaundice could be used in the differential diagnosis.

Sborov, Giges, Plough, and Mandel (6) report a case of post-hepatic jaundice in which ACTH did not induce a lowering of hyperbilirubinemia.

Johnson and Doenges (7), in two cases of hepatitis with hepatocanalicular jaundice, observed a rapid drop of serum bilirubin following the administration of ACTH. In one of these patients who had a common duct drainage the drop was concomitant with the reestablishment of biliary flow. Although they do not report results in cases of post-hepatic jaundice, they suggest that the hormone could be useful in differential diagnosis as it would not influence hyperbilirubinemia in this type of icterus.

According to Patterson, Dingman, Schwachman, and Thorn (8), cortisone has a choleretic action which could explain the lowering of the serum bilirubin values.

Other investigators (4) include the increase in biliary flow among the factors which could explain the drop of hyperbilirubinemia in cases of hepatitis.

Our findings in the first case of post-hepatic jaundice who received cortisone prompted us to study the influence of steroids in cases of complete biliary obstruction. This patient showed a clear-cut drop of serum bilirubin in two periods in which he received cortisone, despite a carcinoma

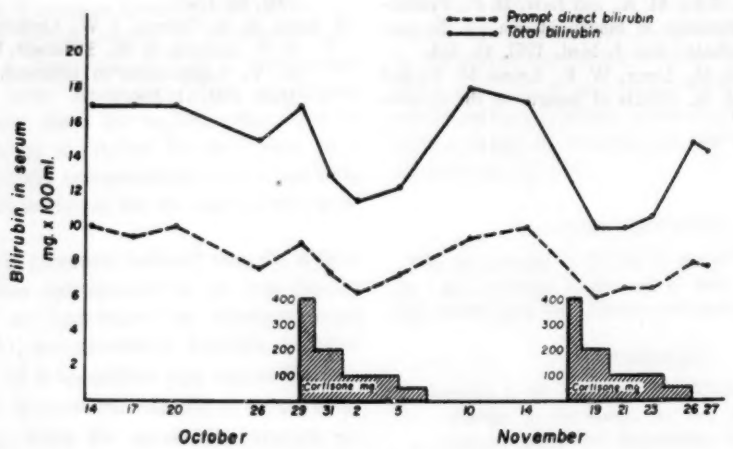


FIG. 1. INFLUENCE OF CORTISONE ON SERUM BILIRUBIN IN POST-HEPATIC JAUNDICE WITH COMPLETE OBSTRUCTION DUE TO CARCINOMA OF THE HEAD OF THE PANCREAS

Case 1. 38 year-old male. Hospital number 53/15891.

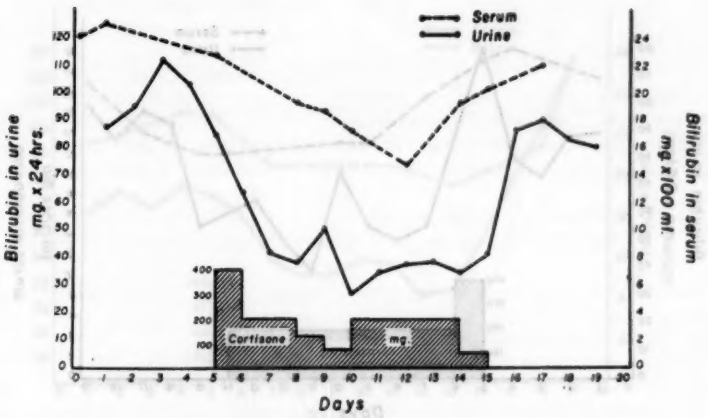


FIG. 2. INFLUENCE OF CORTISONE ON SERUM AND URINE BILIRUBINS
Case 2. R.E., 68 year-old female. Hospital number 56/4746.
The values for urine bilirubin in this and the following figures are those obtained by Jendrassik and Gróf's method (15).

of the head of the pancreas with complete obstruction of the common bile duct, as shown at operation (Figure 1).

These results suggested that the choleretic effect, if present, was not an important factor in the reduction of serum bilirubin values. Moreover they showed that jaundice, even when due to biliary obstruction, was influenced by cortisone.

Chalmers and associates (9) have recently reported their findings with a four-day ACTH test in the differential diagnosis of jaundice. They observed a definite overlapping of effects, thereby failing to confirm the usefulness of the test. Among their patients with biliary obstruction, two showed more than a one-third drop of serum bilirubin despite no detectable urinary urobilinogen throughout the observation period. It is interesting to call attention, in relation to what will be reported later, to the observations of these investigators in chronic hyperbilirubinemia. They found that the drop in bilirubin in the majority of patients was presumably not a result of increased

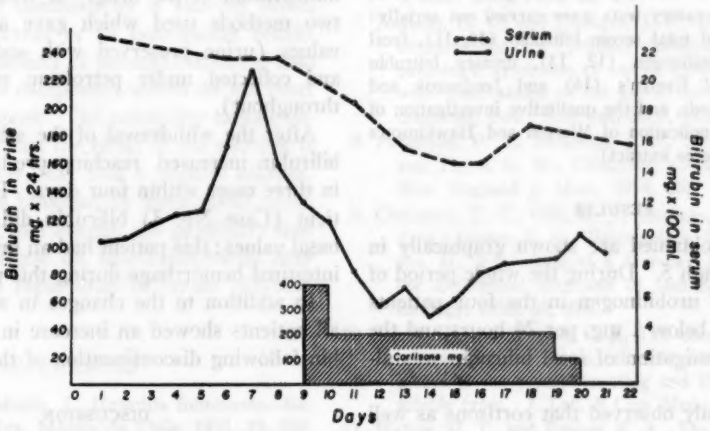


FIG. 3. INFLUENCE OF CORTISONE ON SERUM AND URINE BILIRUBINS
Case 3. L.S., 57 year-old male. Hospital number 56/9226.

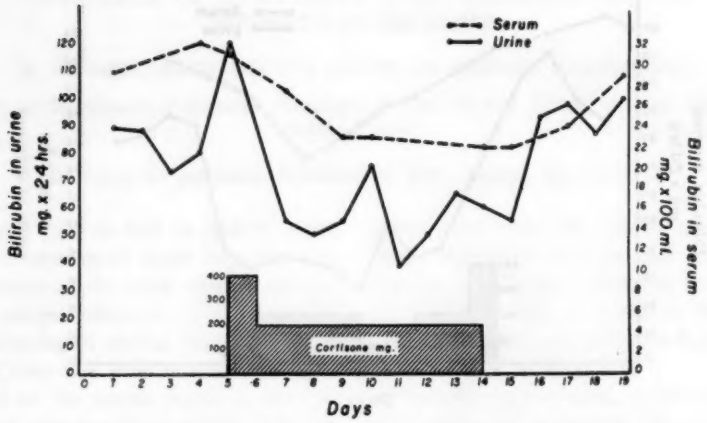


FIG. 4. INFLUENCE OF CORTISONE ON SERUM AND URINE BILIRUBINS
Case 4. E.C., 63 year-old female. Hospital number 56/8648.

choleresis, because it was accompanied by a decrease rather than an increase in fecal urobilinogen output.

METHODS OF STUDY

For the purpose of investigating the effect of steroids on hyperbilirubinemia of cases in which choleresis could not have a role, we chose cases of post-hepatic [1] jaundice with complete biliary obstruction. These patients were selected because they had sustained fecal acholia (absence of fecal urobilinogen) due to complete biliary obstruction. Three received cortisone and one prednisolone by the oral route for periods of 9 to 10 days. The dosage is indicated in Figures 2 through 5.

During the basal observation period, during the administration of the steroid, and following its withdrawal, the following laboratory tests were carried out serially: prompt direct and total serum bilirubin (10, 11), fecal and urinary urobilinogen (12, 13), urinary bilirubin with Malloy and Evelyn's (14) and Jendrassik and Gróf's (15) methods, and the qualitative investigation of fecal bilirubin (application of Watson and Hawkinson's method (16) to feces extract).

RESULTS

The results obtained are shown graphically in Figures 2 through 5. During the whole period of study the fecal urobilinogen in the four patients was constantly below 1 mg. per 24 hours and the qualitative investigation of fecal bilirubin was always negative.

It can be easily observed that cortisone as well as prednisolone, given in doses similar to those used by the authors in the treatment of acute benign hepatitis, produced maximal drops of serum bilirubin of 28 to 34 per cent from the initial value. This drop, although evident, is less than that observed by the present investigators in acute hepatitis during the first four days of administration of cortisone (55.7 per cent).

The drop of serum bilirubin in our cases of complete biliary obstruction was accompanied by persistence of acholia and, in contrast to what would be expected, by a reduction of the urinary excretion of bilirubin. This reduction, although variable, was observed in all four cases during the administration of the drug. It was shown by the two methods used which gave almost identical values (urine preserved with sodium carbonate and collected under petroleum ether was used throughout).

After the withdrawal of the steroid the serum bilirubin increased, reaching pre-treatment levels in three cases within four days. In the other patient (Case No. 3) bilirubin did not reach the basal values; this patient had an important gastrointestinal hemorrhage during this period.

In addition to the changes in serum bilirubin, all patients showed an increase in urinary bilirubin following discontinuation of the steroid.

DISCUSSION

The results here reported demonstrate the effect of cortisone and prednisolone on the hyperbilirubi-

[1] Obstructive jaundice of other classifications.

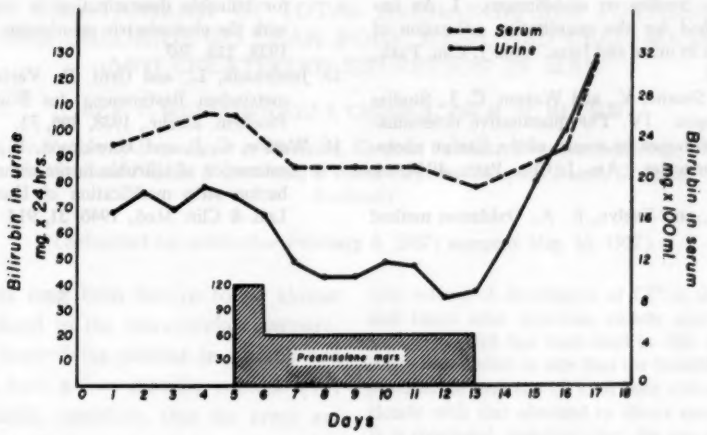


FIG. 5. INFLUENCE OF PREDNISOLONE ON SERUM AND URINE BILIRUBINS
Case 5. R.M., 73 year-old female. Hospital number 56/12133.

nemia of post-hepatic jaundice with complete obstruction. The lowering of the serum bilirubin values, although less marked than that observed in acute hepatitis during similar periods, is clearly apparent.

Concomitantly with the lowering of serum bilirubin, a regular decrease of urine bilirubin was observed.

These findings permit the exclusion of an increase in biliary flow or in urinary excretion of bilirubin as the causes for the lowering of hyperbilirubinemia. This could then be explained by a diminution of bilirubinogenesis. The formation of bilirubin could diminish by a slowing down of red cell destruction or of hemoglobin breakdown. The metabolism of hemoglobin could also deviate to colorless compounds which do not react with the diazo reagent. The possibility cannot entirely be ruled out that the effect of the steroids is exerted on the liver, and that a hepatic bilirubinogenesis which is inhibited by them could exist.

The results reported suggest the possibility that the effect of cortisone on bilirubinogenesis may play a role in the rapid drop of serum bilirubin in acute hepatitis.

REFERENCES

1. Ducci, H., and Motlis, J., Hepatitis fulminante—Recuperación. Rev. Médica de Chile, 1951, **79**, 590.
2. Evans, A. S., Sprinz, H., and Nelson, R. S., Adrenal hormone therapy in viral hepatitis. I. The effect of ACTH in the acute disease. Ann. Int. Med., 1953, **38**, 1115.
3. Siegenthaler, W., and Suter, L., Zur Behandlung der Hepatitis epidemica mit Cortison. Schweiz. med. Wchnschr., 1955, **85**, 1051.
4. Huber, T. E., and Wiley, A. T., Cortisone in the treatment of infectious hepatitis. Ann. Int. Med., 1955, **42**, 1011.
5. Solem, J. H., and Olsen, A., The course of icterus index and prothrombin value during corticotropin treatment of acute hepatitis and obstructive jaundice. A preliminary report. Acta med. Scandinav., 1953, **146**, 281.
6. Sborov, V. M., Giges, B., Plough, I. C., and Mandel, W., ACTH therapy in acute viral hepatitis. J. Lab. & Clin. Med., 1954, **43**, 48.
7. Johnson, H. C., and Doenges, J. P., Intrahepatic obstructive jaundice (primary cholestasis), a clinicopathologic syndrome of varied etiology: a review with observations of the use of corticotropin as a diagnostic tool. Ann. Int. Med., 1956, **44**, 589.
8. Patterson, P. R., Dingman, J. F., Schwachman, H., and Thorn, G. W., Choleretic action of cortisone. New England J. Med., 1954, **251**, 502.
9. Chalmers, T. C., Gill, R. J., Jernigan, T. P., Svec, F. A., Jordan, R. S., Waldstein, S. S., and Knowlton, M., Evaluation of a four-day ACTH test in the differential diagnosis of jaundice. Gastroenterology, 1956, **30**, 894.
10. Ducci, H., and Watson, C. J., The quantitative determination of the serum bilirubin with special reference to the prompt-reacting and the chloroform-soluble types. J. Lab. & Clin. Med., 1945, **30**, 293.
11. Malloy, H. T., and Evelyn, K. A., The determination of bilirubin with the photoelectric colorimeter. J. Biol. Chem., 1937, **119**, 481.

12. Watson, C. J., Studies on urobilinogen. I. An improved method for the quantitative estimation of urobilinogen in urine and feces. Am. J. Clin. Path., 1936, **6**, 458.
13. Schwartz, S., Sborov, V., and Watson, C. J., Studies of urobilinogen. IV. The quantitative determination of urobilinogen by means of the Evelyn photoelectric colorimeter. Am. J. Clin. Path., 1944, **14**, 598.
14. Malloy, H. T., and Evelyn, K. A., Oxidation method for bilirubin determination in bile and meconium with the photoelectric colorimeter. J. Biol. Chem., 1938, **122**, 597.
15. Jendrassik, L., and Gróf, P., Verfahren zur photometrischen Bestimmung des Bilirubins im Harn. Biochem. Ztschr., 1938, **296**, 71.
16. Watson, C. J., and Hawkinson, V., Semiquantitative estimation of bilirubin in the urine by means of the barium-strip modification of Harrison's tests. J. Lab. & Clin. Med., 1946, **31**, 914.

THE RELATIONSHIP OF TOTAL EXCHANGEABLE POTASSIUM AND CHLORIDE TO LEAN BODY MASS, RED CELL MASS AND CREATININE EXCRETION IN MAN

By F. P. MULDOWNEY,[1] J. CROOKS, AND M. M. BLUHM

(From the University Department of Medicine, Gardiner Institute, Western Infirmary, Glasgow, Scotland and Physics Department, Western Regional Hospital Board, Glasgow, Scotland)

(Submitted for publication February 8, 1957; accepted May 16, 1957)

Potassium has long been known to be almost completely confined to the intracellular compartment, and the neutral fat present in muscle has been shown to have a low chloride content (1). It seemed probable, therefore, that the error associated with predictions of total exchangeable electrolyte from total body weight would diminish if the variable of depot fat was eliminated by the use of lean body mass as a reference standard. A close correlation of lean body mass with red cell mass (2) and urinary creatinine (3) has previously been shown. Accordingly, measurements of total exchangeable chloride and potassium have been correlated with lean body mass, red cell mass and creatinine excretion in a series of normal subjects.

MATERIALS AND METHODS

Sixteen normal males and fourteen normal females were studied, their ages ranging from 18 to 81 years and their body weights from 44.5 to 100.2 Kg. They were selected to include both lean and obese subjects, the range of body fat being 5 to 50.8 per cent of total body weight.

Total exchangeable potassium (K_e) measurements were carried out according to the method of Corsa, Olney, Steenburg, Ball, and Moore (4), four spot urine specimens being collected between twenty-two and twenty-six hours after injection. The urinary potassium concentration was estimated by flame photometry. Since the short half-life of Cl^{38} precluded its use, Br^{82} was used to measure the chloride space, one plasma sample being obtained after a period of equilibration of twenty-two to twenty-four hours (5). Plasma chloride was measured by the method of Van Slyke (6), and no correction was made for the Donnan equilibrium. The total exchangeable chloride (Cl_e) was derived from the equation:

Total exchangeable chloride (Cl_e) (mEq.)
= chloride space (liters)
× serum chloride (mEq. per liter)

The volume of distribution of Cl^{38} in man, two and one-half hours after injection, closely approximates that of Br^{82} (7) which has been used in this study. Moreover, it has been shown in rats that the bromide dilution method provides an estimate of total body chloride which agrees closely with that obtained by direct carcass analysis (8). It is concluded, therefore, that the use of Br^{82} provides a valid estimate of Cl_e which is in turn a true measure of total body chloride. One hundred fifty μc. of K^{42} (potassium chloride), irradiated as potassium carbonate, and 25 μc. of Br^{82} (sodium bromide), irradiated as ammonium bromide, were used irrespective of body weight. The radiation dose for a person weighing 70 Kg. is 0.11 rad[2] due to K^{42}, and 0.06 rad due to Br^{82}.

Values for lean body mass were derived from the Pace-Rathbun formula (9): Lean body mass = Total body water × 100/73. Total body water was derived from the antipyrine space, which was obtained from the antipyrine concentration in plasma using the method of Brodie, Axelrod, Soberman, and Levy (10). The plasma concentration of antipyrine was corrected for plasma proteins which were assumed to average 7 per cent of plasma volume. Values of lean body mass in Subjects 12, 14, 15, 16, 27 and 30 were derived from measurements of red cell mass, employing the correlation previously established (2).

To derive red cell mass, plasma volume measurements were performed by means of the Evans blue (cellulose extraction) method (11), as modified by Muldowney (2). Hematocrit figures were obtained with Wintrobe tubes spun at 3,000 r.p.m. for 55 minutes. A correction for trapped plasma was made according to the figures of Chaplin and Mollison (12). The true venous hematocrit was then corrected by the factor 0.91 to calculate total body hematocrit (13). Red cell mass was calculated from the plasma volume as follows:

Red cell mass = plasma volume
× (total body hematocrit)/(100 − total body hematocrit).

Measurements of 24-hour urinary creatinine excretion were performed according to the method of Folin (14) as described by Hawk, Oser and Summerson (15).

[1] Present address: St. Vincent's Hospital, Dublin, Eire.

[2] One rad is equal to an absorbed dose of 100 ergs per gram of tissue.

TABLE I

The data (males)

Subject	Age	Height	Weight	Anti-pyrine space	Lean body mass	Fat % of body weight	Red cell mass	Exchange-able K	Exchange-able Cl	Urinary creatinine
		cm.	*Kg.*	*liters*	*Kg.*		*ml.*	*mEq.*	*mEq.*	*mg./day*
1	22	168.0	61.3	35.6	48.8	20.4	1,818	2,960	1,710	1,485
2	27	183.0	72.7	45.5	58.5	14.3	2,090	3,680	2,004	1,852
3	22	177.0	68.2	45.1	56.15	9.4	2,060	3,060	2,020	2,110
4	36	175.5	97.4	45.0	61.6	36.7	2,110	3,560	2,270	2,060
5	22	177.0	65.4	35.45	48.6	25.6	1,740	3,030	1,850	1,970
6	28	184.0	71.8	44.6	61.1	15.0	2,223	3,530	2,162	
7	22	192.0	82.0	50.4	71.7	15.7	2,590	4,240	3,015	2,330
8	22	169.0	65.0	37.95	52.0	20.0	1,748	2,910	1,930	1,480
9	23	178.0	65.5	42.5	58.2	11.0			2,185	1,562
10	25	183.0	87.2	53.0	72.6	16.8	2,480		2,450	1,660
11	27	175.0	57.3	36.4	49.8	13.0	1,795	3,160	1,950	
12	33	178.0	77.2		59.5*	23.0	2,100	3,390		1,870
13	43	164.5	61.0	41.9	57.45	6.0	2,035		1,975	
14	29	168.0	55.8		53.0*	5.0	1,890	2,700	1,805	1,213
15	33	165.0	63.6		51.1*	19.7	1,830	2,650	1,776	1,580
16	59	183.0	82.7		55.1*	33.5	1,960	2,720	1,928	1,380

* Derived from red cell mass.

RESULTS

The complete data are given in Tables I and II and the statistical analysis is summarized in Table III.

The correlation of K_e with total body weight (Figure 1) was poor ($r = 0.45$) and the 95 per cent confidence limits were very wide ($\pm$ 53 per cent of mean). When correlating K_e with lean body mass (Figure 2), red cell mass (Figure 3) and 24-hour urinary creatinine (Figure 4), it was found that the correlation coefficients were equally good ($r = 0.90$ to 0.91), and that in each case the 95 per cent confidence limits agreed fairly closely ($\pm$ 25 per cent to 28 per cent of mean).

The correlation of Cl_e with total body weight (Figure 5) was better than that of K_e, the correlation coefficient ($r = 0.58$) being higher, and the 95 per cent confidence limits being narrower ($\pm$ 29 per cent of mean). In the correlation of Cl_e with lean body mass (Figure 6) and red cell mass (Figure 7), the correlation coefficients ($r = 0.93$) were again equally good, and the 95 per cent confidence limits ($\pm$ 14 per cent to 15 per cent of mean) agreed closely. The correlation of 24-hour urinary

TABLE II

The data (females)

Subject	Age	Height	Weight	Anti-pyrine space	Lean body mass	Fat % of body weight	Red cell mass	Exchange-able K	Exchange-able Cl	Urinary creatinine
		cm.	*Kg.*	*liters*	*Kg.*		*ml.*	*mEq.*	*mEq.*	*mg./day*
17	45	140.0	83.6	30.0	41.1	50.8	1,509	1,840	1,580	646
18	55	156.5	82.7	35.4	48.5	41.4	1,840		1,827	
19	43	153.0	86.4	33.0	45.2	47.7	1,688	2,670	1,730	
20	31	168.0	60.0	31.7	43.5	27.4	1,620	2,340	1,525	
21	57	158.0	93.6	38.0	52.0	44.5	1,868	2,400	1,980	890
22	38	155.0	70.5	33.5	45.9	34.8	1,695	2,100		1,168
23	81	152.0	44.5	24.3	33.3	25.2	1,200	1,190	1,248	527
24	55	156.0	66.0	33.1	45.3	31.4	1,605		1,680	1,230
25	18	173.0	100.2	45.0	61.5	39.7	2,080	2,910	2,440	1,310
26	50	157.5	57.2	33.0	45.2	26.7	1,695		1,728	
27	51	151.0	50.0		36.65*	16.0	1,355	1,800		1,262
28	52	152.0	63.6	34.3	47.0	26.0	1,735	2,040	1,720	1,024
29	37	170.0	55.0	33.42	45.8	16.7	1,760		1,830	
30	66	162.5	54.0		42.1*	22.0	1,535	1,610	1,369	810

* Derived from red cell mass.

TABLE III

K_e or Cl_e	Reference standard	r	95 per cent confidence limits	95 per cent confidence limits	Equation of regression line
			*mEq.**	*% of mean*	
K_e	Total body weight (TBW)	0.45	±1,440	±53	$K_e = 21.71(TBW) + 1,181.5$
K_e	Lean body mass (LBM)	0.90	± 680	±25	$K_e = 73.98(LBM) - 1,063.3$
K_e	Red cell mass (RCM)	0.91	± 670	±25	$K_e = 2.21(RCM) - 1,341.1$
K_e	Urinary creatinine (C)	0.90	± 740	±28	$K_e = 1.36(C) + 745.6$
Cl_e	Total body weight (TBW)	0.58	± 550	±29	$Cl_e = 14.35(TBW) + 902.5$
Cl_e	Lean body mass (LBM)	0.93	± 270	±14	$Cl_e = 37.19(LBM) - 23.2$
Cl_e	Red cell mass (RCM)	0.93	± 290	±15	$Cl_e = 1.13(RCM) - 204.5$
Cl_e	Urinary creatinine (C)	0.72	± 630	±33	$Cl_e = 0.58(C) + 118.6$

* Taken about the mean value of the reference standard.

creatinine (Figure 8) showed a lower degree of correlation ($r = 0.72$) and wide 95 per cent confidence limits (± 33 per cent of mean) were obtained.

The standard error of each individual measurement of K_e and Cl_e was ascertained and was found to be ± 5 per cent for K_e and ± 3 per cent for Cl_e. These represent errors in the physical and chemical methods involved, the largest contribution be-

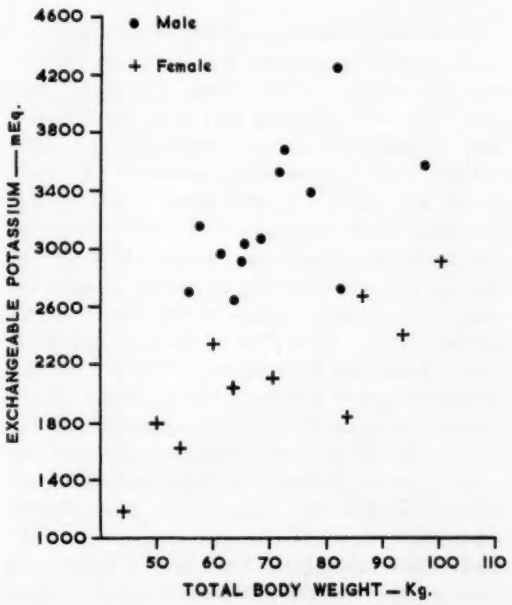


FIG. 1. CORRELATION OF TOTAL EXCHANGEABLE POTASSIUM WITH TOTAL BODY WEIGHT

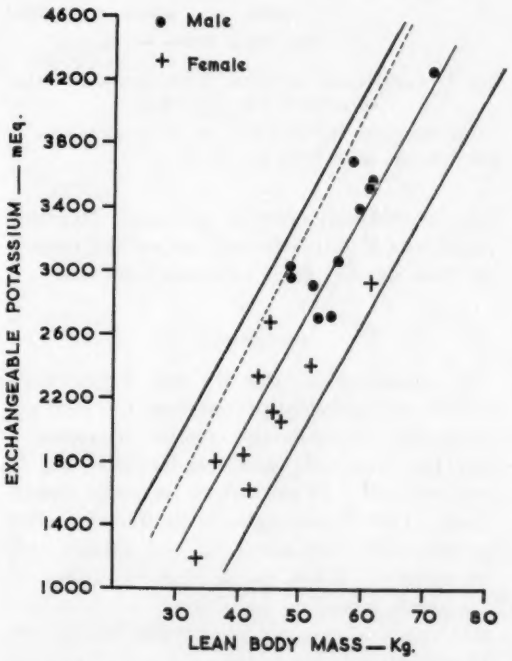


FIG. 2. CORRELATION OF TOTAL EXCHANGEABLE POTASSIUM WITH LEAN BODY MASS

The continuous lines are the regression line of K_e on lean body mass and its 95 per cent confidence limits. The interrupted line is the regression line derived from the data of Ikkos and associates (17).

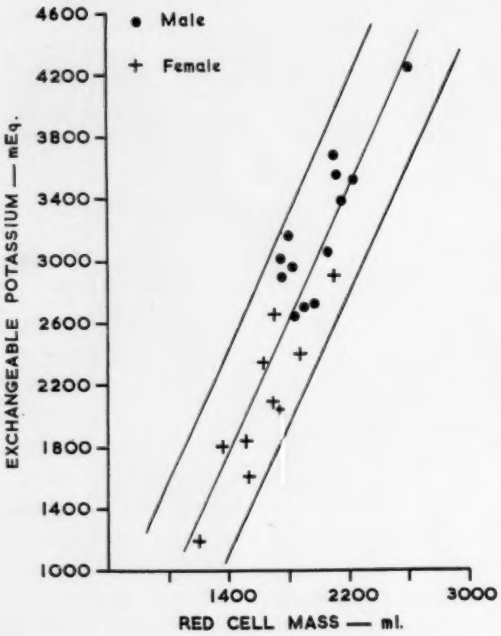


FIG. 3. CORRELATION OF TOTAL EXCHANGEABLE POTASSIUM WITH RED CELL MASS

The regression line of K_e on red cell mass and its 95 per cent confidence limits are shown.

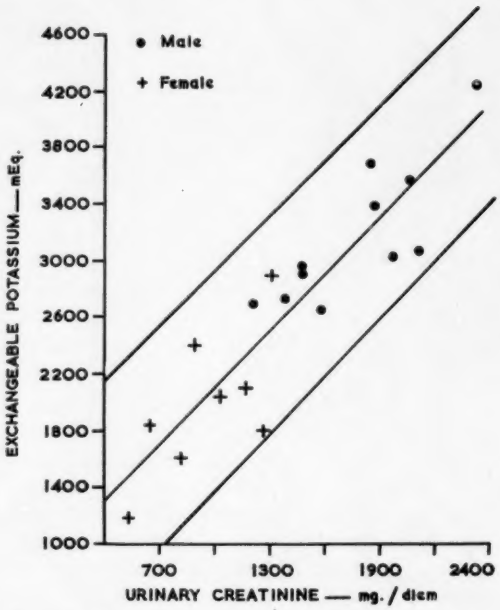


FIG. 4. CORRELATION OF TOTAL EXCHANGEABLE POTASSIUM WITH URINARY CREATININE

The regression line of K_e on urinary creatinine and its 95 per cent confidence limits are shown.

ing the statistical error in counting. Repeated estimations of lean body mass and red cell mass in the same subject agreed to within 4 per cent.

DISCUSSION

The results show that the use of total body weight as a standard of reference for total exchangeable potassium and chloride is unsatisfactory, the 95 per cent confidence limits being ± 53 per cent and ± 29 per cent of the mean, respectively. Our figures agree with those of other workers when both males (4) and females (16) are included. When, on the other hand, total exchangeable potassium and chloride are correlated with lean body mass, the relationship is closer, the 95 per cent confidence limits being ± 25 per cent and ± 14 per cent of the mean, respectively. Furthermore, the relationship is the same for both males and females, the apparent sex difference, based on total body weight, being due to the greater fat content of the female subjects.

These results are supported by experiments carried out by other workers both in human subjects and in animals. Thus, Ikkos, Ljunggren, and Luft (17) in Sweden, as part of an investigation into the relation between extracellular and intracellular water in acromegaly, measured total body water and K_e in a control group of 33 normal subjects. From their data it is possible to derive antipyrine space measurements and, after correcting for the water content of plasma, to estimate lean body mass by means of the Pace-Rathbun formula (9). When this is done, a regression line of K_e on lean body mass is obtained which has been included in Figure 2, the coefficient of the correlation being 0.92. This regression line lies within the 95 per cent confidence limits of the present series, although a slight systematic difference is apparent. Data for K_e, Cl_e and total body water given by Corsa and colleagues (4) and Ikkos, Ljunggren, Luft, and Sjögren in a later paper (18) are consistent with the present results when lean body mass is derived from their values for total body water.

Carcass analysis in rats provides direct evidence of a close correlation of total body potassium and total body chloride with lean body mass (8). Weir (19) had previously demonstrated that total exchangeable chloride was directly related to the lean carcass in dogs, while Hastings and Eichelberger (1) commented on the necessity for analyzing fat-free muscle to allow comparison of the electrolyte content of muscle samples from different animals, or even from different muscles of the same animal. This evidence suggested, therefore, that chloride is confined to lean tissue, and is not present in depot fat. Forbes and Lewis (20), however, have analyzed two human cadavers and found an appreciable chloride content in adipose tissue. This appears to conflict with the results of the present series, and with the data from animals quoted above. The discrepancy is one of definition, however. It must be clearly stated that depot fat, as referred to here and in the preceding communication (2), refers only to the lipid content of adipose tissue. Keys and Brozek (21) estimate that lipids form 62 per cent, water 31 per cent and cell solids 7 per cent of adipose tissue. It is important to note that these relative proportions of water and cell solids are similar to those obtained in muscle, and that the non-lipid portion of adipose tissue is therefore included in the estimate of lean body mass derived from total body water. One would expect that adipose tissue as a whole should contain a percentage of chloride and potassium in proportion to its non-lipid content.

In view of the high degree of correlation which has been shown to exist between lean body mass and red cell mass (2) it is not surprising to find a similar close relationship between red cell mass and both K_e and Cl_e. Provided hematological abnormality can be excluded, it is possible that this relationship may have an application in states of acute electrolyte disturbance which are so often associated with changes in total body water which invalidate the determination of lean body mass by the antipyrine dilution method. Further work,

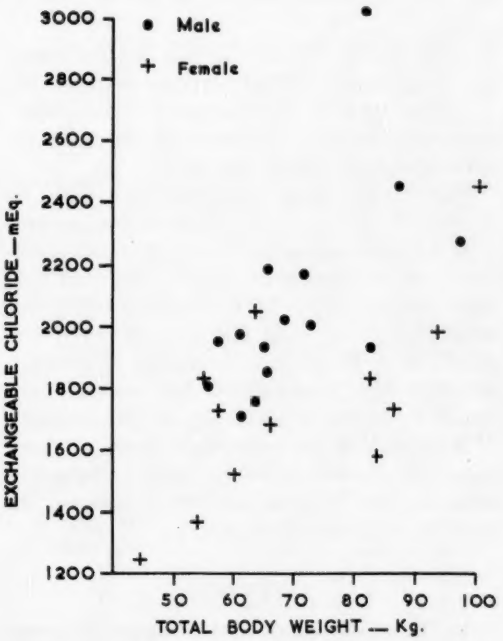


FIG. 5. CORRELATION OF TOTAL EXCHANGEABLE CHLORIDE WITH TOTAL BODY WEIGHT

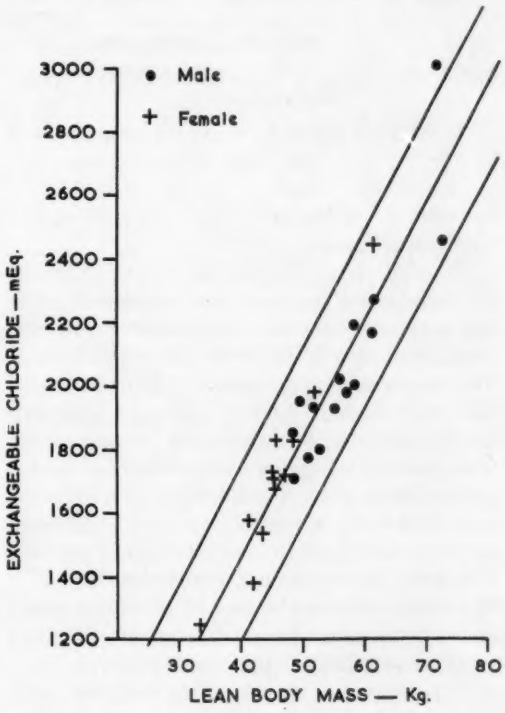


FIG. 6. CORRELATION OF TOTAL EXCHANGEABLE CHLORIDE WITH LEAN BODY MASS

The regression line of K_e on lean body mass and its 95 per cent confidence limits are shown.

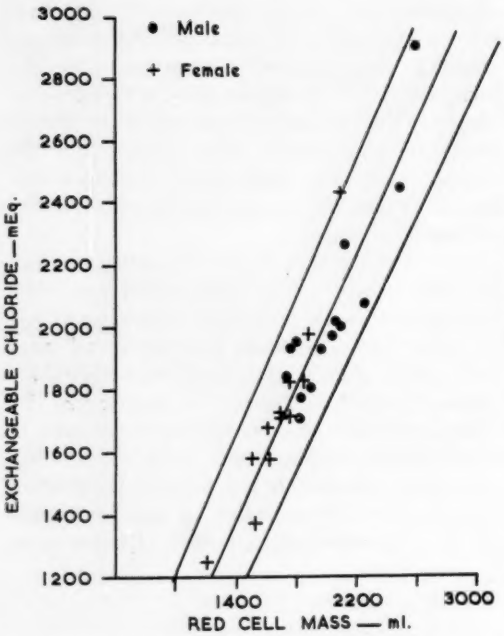


FIG. 7. CORRELATION OF TOTAL EXCHANGEABLE CHLORIDE WITH RED CELL MASS

The regression line of K_e on red cell mass and its 95 per cent confidence limits are shown.

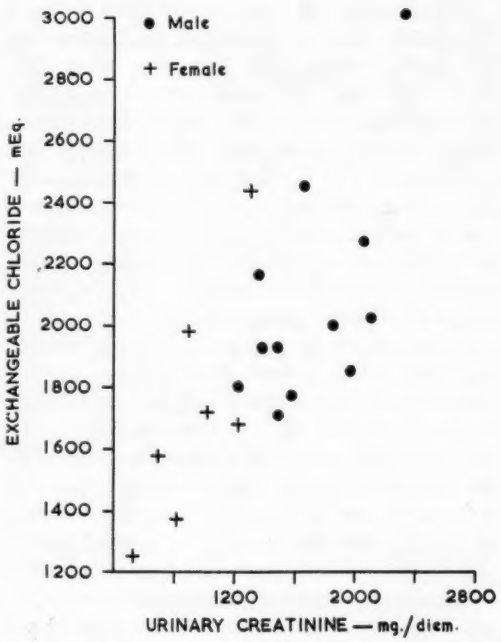


FIG. 8. CORRELATION OF TOTAL EXCHANGEABLE CHLORIDE WITH URINARY CREATININE

however, is required to evaluate the place of this relationship in such states.

The high correlation between K_e and creatinine excretion found by Corsa and associates (4) in five male subjects has been confirmed in this larger series containing both males and females. The closeness of this correlation was unexpected since only single 24-hour specimens of urine, primarily collected for electrolyte measurements, were used for creatinine estimations and dietary protein intake was not controlled. This technique may result in the occasional large errors mentioned by Miller and Blyth (3) and could account for the lean body mass–creatinine correlation ($r = 0.73$, 95 per cent confidence limits $\pm$ 28 per cent of mean) of this series as compared with that of Miller and Blyth ($r = 0.826$, 90 per cent confidence limits $\pm$ 13.1 per cent of mean). This, however, could not wholly explain why Cl_e did not correlate any better with creatinine than with total body weight. The value of creatinine excretion as a standard of reference for K_e lies in its relative ease of estimation, particularly where facilities for measuring lean body mass or red cell mass are not available. It suffers from the disadvantage that our knowledge of disturbances of creatinine metabolism in pathological states is incomplete.

The assessment of electrolyte disturbance by means of serum electrolyte concentration has been shown to be fallacious insofar as serum concentration does not necessarily reflect changes in total body content (*22*). It is highly desirable that measurements of total exchangeable electrolyte should be employed in such studies. The application of these measurements has, however, been limited by the lack of a suitable reference standard. It is hoped that the provision of these narrower ranges of normal values for total exchangeable potassium and chloride may be of value in the study of electrolyte disturbances.

SUMMARY

1. The correlation of total exchangeable potassium (K_e) and chloride (Cl_e) with total body weight in normal subjects is poor.

2. A close correlation of K_e and Cl_e with lean body mass and red cell mass in normal subjects is established.

3. There is a close relationship between 24-hour urinary creatinine and K_e, but not between 24-hour urinary creatinine and Cl_e.

4. The correlations hold throughout a wide range of age and body weight and are the same for both males and females.

ACKNOWLEDGMENTS

The authors are grateful for the encouragement and advice of Professor E. J. Wayne, and to Dr. R. A. Robb for the statistical analysis of the results. The technical assistance of Miss E. Macdonald and Mr. J. McShera is gratefully acknowledged.

REFERENCES

1. Hastings, A. B., and Eichelberger, L., The exchange of salt and water between muscle and blood. I. The effect of an increase in total body water produced by the intravenous injection of isotonic salt solutions. J. Biol. Chem., 1937, **117**, 73.
2. Muldowney, F. P., The relationship of total red cell mass to lean body mass in man. Clin. Sc., 1957, **16**, 163.
3. Miller, A. T., Jr., and Blyth, C. S., Estimation of lean body mass and body fat from basal oxygen consumption and creatinine excretion. J. Applied Physiol., 1952, **5**, 73.
4. Corsa, L., Jr., Olney, J. M., Jr., Steenburg, R. W., Ball, M. R., and Moore, F. D., The measurement of exchangeable potassium in man by isotope dilution. J. Clin. Invest., 1950, **29**, 1280.
5. Bradley, J. E. S., Davidsson, D., MacIntyre, I., and Rapoport, A., Estimation of extracellular volume using ^{82}Br (abstract). Biochem. J., 1956, **62**, 33.
6. Van Slyke, D. D., The determination of chlorides in blood and tissues. J. Biol. Chem., 1923, **58**, 523.
7. Gamble, J. L., Jr., Robertson, J. S., Hannigan, C. A., Foster, C. G., and Farr, L. E., Chloride, bromide, sodium and sucrose spaces in man. J. Clin. Invest., 1953, **32**, 483.
8. Cheek, D. B., and West, C. D., An appraisal of methods of tissue chloride analysis: the total carcass chloride, exchangeable chloride, potassium and water of the rat. J. Clin. Invest., 1955, **34**, 1744.
9. Pace, N., and Rathbun, E. N., Studies on body composition. III. The body water and chemically combined nitrogen content in relation to fat content. J. Biol. Chem., 1945, **158**, 685.
10. Brodie, B. B., Axelrod, J., Soberman, R., and Levy, B. B., The estimation of antipyrine in biological materials. J. Biol. Chem., 1949, **179**, 25.
11. Bedwell, G. A., Patterson, J., and Swale, J., The estimation of Evans blue in plasma. J. Clin. Path., 1955, **8**, 61.
12. Chaplin, H., Jr., and Mollison, P. L., Correction for plasma trapped in the red cell column of the hematocrit. Blood, 1952, **7**, 1227.
13. Chaplin, H., Jr., Mollison, P. L., and Vetter, H., The body/venous hematocrit ratio: its constancy over a wide hematocrit range. J. Clin. Invest., 1953, **32**, 1309.
14. Folin, O., On the determination of creatinine and creatine in urine. J. Biol. Chem., 1914, **17**, 469.
15. Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical Physiological Chemistry, 12th ed. London, J. & A. Churchill, Ltd., 1951, p. 840.
16. Aikawa, J. K., Harrell, G. T., and Eisenberg, B., The exchangeable potassium content of normal women. J. Clin. Invest., 1952, **31**, 367.
17. Ikkos, D., Ljunggren, H., and Luft, R., The relation between extracellular and intracellular water in acromegaly. Acta. endocrinol., 1956, **21**, 211.
18. Ikkos, D., Ljunggren, H., Luft, R., and Sjögren, B., Content and distribution of potassium and chloride in adults. Metabolism, 1955, **4**, 231.
19. Weir, E. G., Further observations on total chloride content; the relationship between body fat and body chloride. Am. J. Physiol., 1940, **130**, 608.
20. Forbes, G. B., and Lewis, A. M., Total sodium, potassium and chloride in adult man. J. Clin. Invest., 1956, **35**, 596.
21. Keys, A., and Brozek, J., Body fat in adult man. Physiol. Rev., 1953, **33**, 245.
22. Moore, F. D., Bodily changes in surgical convalescence. I. The normal sequence—observations and interpretations. Ann. Surg., 1953, **137**, 289.

CLINICAL SCIENCE

Edited for the Medical Research Society
by W. MELVILLE ARNOTT

Assistant Editors
K. W. DONALD, O. L. WADE, H. E. DE WARDENER

Advisory Board

E. A. CARMICHAEL
R. V. CHRISTIE
R. T. GRANT
J. McMICHAEL
G. W. PICKERING
E. E. POCHIN
E. P. SHARPEY-SCHAFER
JANET VAUGHAN
W. C. WILSON
L. J. WITTS

Volume 16, No. 3. **Issued August, 1957.**

The Effect of Changes in Red Cell and Plasma Volumes on the Estimation of Red Cell Survival. By A. TURNBULL, A. HOPE and D. VEREL.

The Effect of Oral Histamine on the Absorption of Radioactive Vitamin B12 from the Gastro-intestinal Tract. By K. B. TAYLOR and W. H. TAYLOR.

Studies on the Urinary Excretion of Oxalate by Normal Subjects. By H. E. ARCHER, A. E. DORMER, E. F. SCOWEN and R. W. E. WATTS.

The Effects of Diamox on Ammonia Metabolism in Liver Disease. By A. M. DAWSON, J. de GROOTE, W. A. ROSENTHAL and SHEILA SHERLOCK.

The Adaptation of the Relaxed Lungs and Chest Wall to Changes in Volume. By J. BUTLER.

Relationship Between Refraction of the Eye and Nutrition. By P. A. GARDINER and I. MACDONALD.

Some Observations on the Assessment of Drugs in Human Tetanus. By D. R. LAURENCE.

Studies on Lewis' Auricular Flutter of Post-Electrical Stimulatory Origin. By REXFORD KENNAMER, ELEANOR MILES and MYRON PRINZMETAL.

Changes in Iron Metabolism Following Gastrectomy and Other Surgical Operations. By I. McL. BAIRD, D. A. PODMORE and G. M. WILSON.

Serum Lipids in Pregnancy and the Puerperium. By W. C. WATSON.

The Physical Properties of the Lungs in Chronic Cor Pulmonale. By J. D. S. HAMMOND.

The Kinetic Resistance of the Relaxed Respiratory System. By JOHN BUTLER.

The Physical Properties of the Lungs in Relation to the Subdivisions of Lung Volume. By ROBERT MARSHALL.

Acquired Porphyria from a Liver Tumour. By TIO (TIONG HOO), B. LEIJNSE, A. JARRETT and C. RIMINGTON.

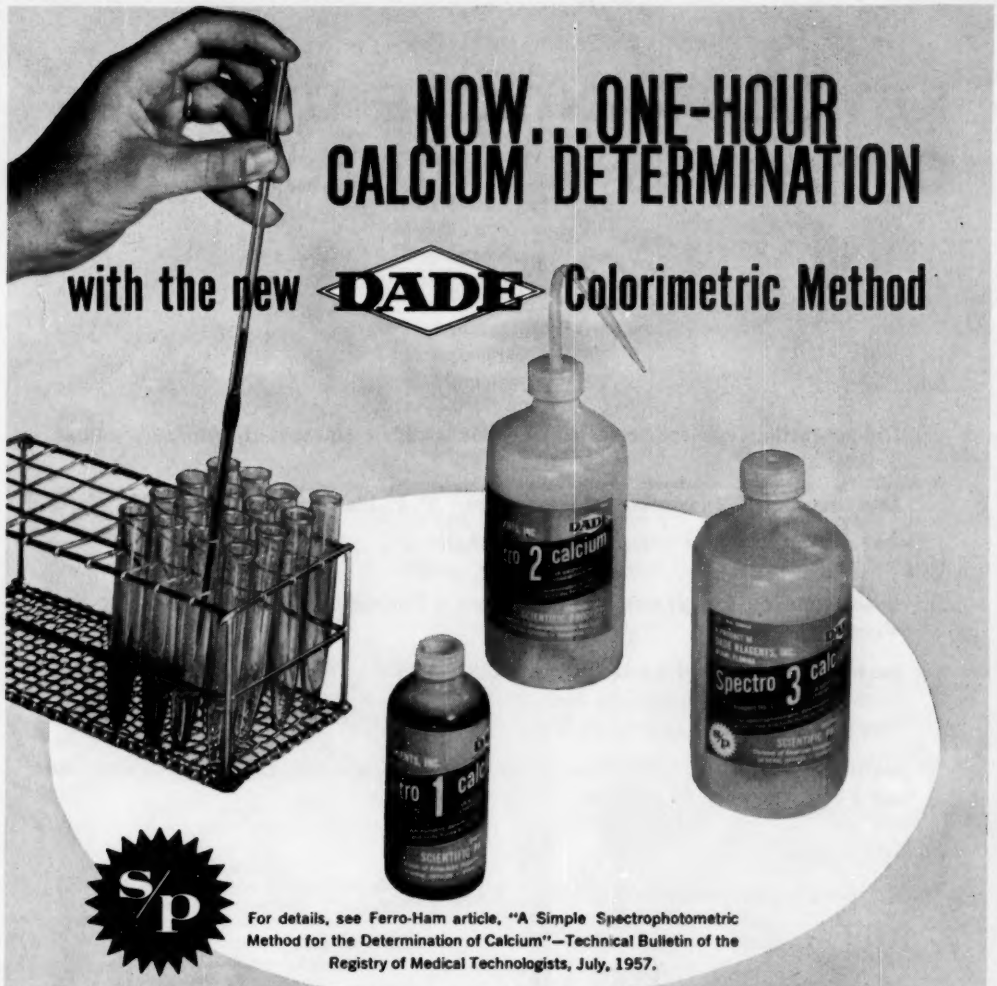
Measurement of the Ventilation-Perfusion Ratio Inequality in the Lung by the Analysis of a Single Expirate. By J. B. WEST, K. T. FOWLER, P. HUGH-JONES and T. V. O'DONNELL.

The Measurement of the Inequality of Ventilation and of Perfusion in the Lung by the Analysis of Single Expirates. By J. B. WEST, K. T. FOWLER, P. HUGH-JONES and T. V. O'DONNELL.

Changes in the Oxygen Content of Femoral Venous Blood and Leg Blood Flow During Leg Exercise in Relation to Cardiac Output Response. By K. W. DONALD, P. N. WORMALD, S. H. TAYLOR and J. M. BISHOP.

Subscription Rate: £3.15.0. per volume.

Publishers: Shaw & Sons Ltd., 7, 8 & 9, Fetter Lane, Fleet Street, London, E. C. 4, England



NOW...ONE-HOUR CALCIUM DETERMINATION

with the new DADE Colorimetric Method

S/P

For details, see Ferro-Ham article, "A Simple Spectrophotometric Method for the Determination of Calcium"—Technical Bulletin of the Registry of Medical Technologists, July, 1957.

Eliminates visual errors... highly reproducible and accurate

This important advancement in determination of calcium saves laboratory time. Based on the Ferro-Ham technique, later improved by the authors, the complete test takes less than one hour.

Visual errors are eliminated through accurate calibrated readings—and the final color remains stable for several days. The method is highly reproducible. Spectrophotometric or colorimetric readings eliminate end point estimation errors found in titration procedures.

Only three reagents are used and only four easy steps are required—

1. Add Chloranilic Acid Reagent to serum—let stand 30 minutes.
2. Centrifuge precipitate—decant supernatant.
3. Add Isopropyl Alcohol Reagent—centrifuge—decant supernatant.
4. Add Versene Reagent—read color in spectrophotometer or colorimeter at 520 mu.

No. 53955—Dade Calcium Determination Kit, sufficient for 75 calcium determinations. $6.75

Scientific Products

DIVISION OF AMERICAN HOSPITAL SUPPLY CORPORATION

New York • Chicago • Kansas City • Minneapolis • Atlanta • Washington • Dallas • Los Angeles • San Francisco

REVUE FRANÇAISE D'ÉTUDES CLINIQUES ET BIOLOGIQUES

JUNE, 1957
Vol. II, No. 6

Editorial

Les interactions nucléocytoplasmiques dans la cellule au repos et pendant la mitose. J. BRACHET.

Evolution des idées concernant les cancers. P. F. DENOIX.

Original Articles

L'isolement du toxoplasme par inoculation à l'animal. G. DESMONTS et LE TAN VINH.

Le traitement palliatif du cancer métastatique du sein par ovariectomie et surrénalectomie totales. Indications fournies par l'étude du métabolisme calcique. W. SMETS, P. DOR, Y. KENIS et H. TAGNON.

Cortisone et syphilis. Résultats de la corticothérapie précédant la cure pénicillinée dans cent vingt cas de syphilis. P. DE GRACIANSKY et CH. GRUPPER.

General Review

La thromboplastino-formation. M. J. LARRIEU.

Technical Note

Modifications pratiques concernant le dosage des antibiotiques en clinique. Y. CHABBERT et H. BOULINGRE.

* * * * *

Subscriptions, U.S.A. and Canada, $19.00

Special rate for interns and residents, $13.00

Flammarion, 1243 University Street, Montreal

AEL INC.

MANUFACTURERS & DESIGNERS OF STANDARD PRECISION RESEARCH AND CLASSROOM ELECTRONIC APPARATUS

Stimulators
Photic Stimulators
Dual Beam Oscilloscopes
Isolators
Regulated Power Supplies
Raster Timers
High Impedance Probes
Intracardiac Phonocatheters
Phonocatheter Amplifier
D.C. Amplifiers
A.C. Amplifiers
Monitor Amplifiers
and Special Devices

For Technical Data Write to Dept. AM
American Electronic Laboratories, Inc.
121 N. 7th Street, Phila. 6, Pa.

NOW OVER 5100 BIOS CHEMICALS

- Pentachloroacetone
- Pentamethonium Bromide
- Pentamidine
- Phenanthridine
- Phenazine Methochloride
- Phenolphthalein Phosphate (Sodium Salt)
- Phenoltetrachlorophthalein
- Phenolthymolphthalein
- 9-Phenylacridine
- 2-Phenylazo-p-cresol
- p-Phenylbenzaldehyde
- α-Phenylglucoside
- Phenylindandione
- 2-Phenylindole
- Phenyllactic Acid
- Phenylmercuric Borate
- Phenylmercuric Lactate
- β-Phenylserine
- Phenylthienyl Ketone

Ask for our new complete catalogue

BIOS Laboratories, Inc.
17 West 60th St. New York 23 N. Y.
PLaza 7-8171

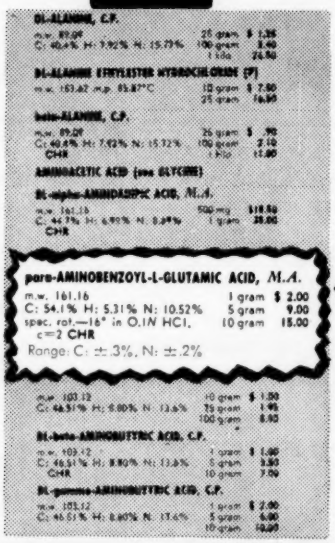
LATEST LITERATURE SUPPLIED

The latest information on Orinase* was made available to the profession during clinical testing period. More than 70,000 requests for literature were received.

*Trademark, Reg. U.S. Pat. Off. — tolbutamide, Upjohn

THE UPJOHN COMPANY
Kalamazoo, Michigan
Upjohn

a new unique service
FOR THE BIOCHEMIST

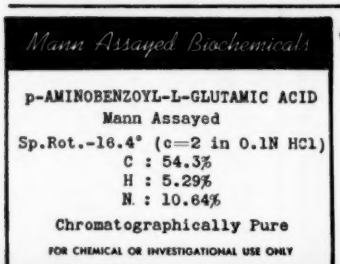


Now, for the first time *Mann Assayed* **BIOCHEMICALS** makes available the complete quality standards for each product. Here indeed is a secure starting point, saving valuable time and effort by providing biochemicals of known purity, and, equally important, of reliably consistent supply. Only *Mann Assayed* **BIOCHEMICALS** provide:

IN THE CATALOG

THEORETICAL VALUES . . . as part of each listing, we include theoretical elementary analysis, exact description and molecular weight.

EXACT RANGE OF DETERMINED VALUES . . . to enable the biochemist to determine in what range the analysis of each biochemical will be.



ON THE LABEL

THE EXACT ANALYSIS of each lot — C,H,N, specific rotation, chromatogram, U.V. absorption, and M.P. when indicated — your final and exact guide — adding a degree of security for the biochemist never before available from any source.

Write, wire or phone today for your copy of the Mann Assayed Biochemicals Catalog 1957 and Price List No. 123.

MANN RESEARCH LABORATORIES, INC.

Dept. K 136 Liberty St., New York 6, N. Y. BEekman 3-5865

LANCASTER PRESS, INC., LANCASTER, PA.